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Factors Influencing The Stability Of Carotenoids In Oil-In-Water Emulsions

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FACTORS INFLUENCING THE STABILITY OF CAROTENOIDS IN OIL-IN-WATER EMULSIONS

A Dissertation Presented

by

Caitlin Suzanne Boon

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DEDICATION

To my loving and supportive family.

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ABSTRACT

FACTORS INFLUENCING THE STABILITY OF CAROTENOIDS IN OIL-IN-WATER EMULSIONS

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Lycopene has recently received interest as an antioxidant in human tissues. These same antioxidant properties present challenges in preventing oxidative degradation within food products. In this research, degradation of lycopene in model emulsion systems was examined to better understand the chemical stability of this potential functional food ingredient.

Lycopene in corn oil or hexadecane was used to make oil-in-water emulsions using small molecule surfactants. Emulsion color loss was used to estimate lycopene loss and was monitored using an integrating sphere. Lipid hydroperoxide and hexanal formation was used to monitor the development of lipid oxidation.

Oxidation and color loss were found to be influenced by surfactant type, with the fastest rates occurring in emulsions stabilized by anionic sodium dodecyl sulfate (SDS) and slower rates occurring in emulsions stabilized by cationic dodecyltrimethylammonium bromide (DTAB) and nonionic Brij 35. Lycopene oxidized

in the presence and absence of unsaturated fatty acids, suggesting that degradation can occur by mechanisms that do not involve lipid oxidation products.

Further understanding of the mechanisms of lycopene degradation was gained by exposing emulsions to light, varied pH, a metal chelator, and a free radical scavenger. Results suggest that transition metal induced oxidation of lycopene may be the predominant mechanism of degradation at low pHs where transition metal solubility is high. At higher pHs, where metal solubility is lower, attack by free radicals was also found to be contributing to lycopene oxidation.

The role of ferric and ferrous species of iron in lycopene degradation was also investigated. In SDS-stabilized emulsions, and in bulk hexadecane, lycopene was found to degrade fastest in the presence of ferric iron. Ferrozine chelation testing show that this degradation is likely due to the reaction of lycopene with ferric ions to produce the ferrous species and a lycopene radical cation. In nonionic, Brij 35 and Tween 20 emulsions, ferrous iron resulted in the most rapid lycopene degradation. While further work is needed to clarify these findings, results from ferrozine analyses suggests that ferrous ions may be oxidized to the ferric species by naturally present hydroperoxides present in the surfactants, and then go on to attack lycopene.

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CHAPTER 1

INTRODUCTION

Carotenoids are natural pigments responsible for the coloration of a variety of plants and animals. In recent years, a number of studies have produced evidence to suggest that consuming carotenoids may provide a variety of health benefits including a reduced incidence of a number of cancers, reduced risk of cardiovascular disease, and improved eye health. Many of these health benefits are thought to be due to the antioxidant activity of carotenoids in certain environments.

Evolving evidence on the health benefits of several carotenoids has sparked interest in incorporating more carotenoids into functional food products. Unfortunately, the same structural attributes of carotenoids that are thought to impart antioxidant health benefits also make these compounds highly susceptible to oxidation. Heat, light, oxygen, extremes in pH, transition metals, and interactions with radical species have all been found to cause destruction of carotenoids.

Given the susceptibility of carotenoids to degradation, particularly once they have been extracted from biological tissues, specially designed systems are needed to protect these compounds when they are used as functional food ingredients. Oil-in-water emulsions may be one system that would offer multiple protection options for the carotenoids. These systems can be engineered to provide antioxidative functions in the aqueous, oil, and interfacial regions. Emulsions may also offer advantages as a food ingredient as they are easily incorporated into food products and can be engineered to maintain their protective properties once dispersed into a food system.

Lycopene is an acyclic carotenoid that has gained popularity as a functional food ingredient in recent years due to a number of studies suggesting potential health benefits from consuming diets rich in this compound. The objective of this work is to determine the potential for using emulsion systems as a protective delivery system for lycopene as a functional food ingredient and to better understand the mechanisms by which lycopene degradation occurs in these systems.

CHAPTER 2

LITERATURE REVIEW

2.1 Health benefits of carotenoids

Carotenoids are tetraterpenes (1) that are characterized by a conjugated system of double bonds with delocalized π-electrons (2) The conjugated double bond system of these compounds imparts pigmentation to a variety of plant and animal life. Being extremely hydrophobic, these compounds are usually found in the core of membranes or other hydrophobic locations (2). Carotenoids can be found in an unbound form or can be bound to fatty acids, glycosides, or proteins (3). They are found in the tissues of plants and algae as well as microorganisms, and they are responsible for the feather and flesh colors of some birds and fish. Aside from coloration, these compounds are also important for plant and animal health. These compounds often function as an antioxidant and play special roles in protection of tissues from damage caused by light and oxygen (4). However, the same properties that make carotenoids useful healthy tissue function, creates challenges in preventing the degradation of carotenoids in food products. With increasing awareness of potential health benefits of certain carotenoids, there has been a surge of interest in creating functional food products containing carotenoid ingredients.

Several carotenoids commonly found in foods are thought to play a role in maintaining bodily functions and preventing disease. Beta-carotene, lycopene, lutein, and zeaxanthin are some of the most well known carotenoids considered to have health benefits. Each of these compounds and their proposed benefits are discussed below.

2.1.1 Beta-carotene

Beta-carotene ($C_{40}H_{56}$) (**Figure 2.1**) is one of the major carotenoids present in the diet (5). It is found in a variety of orange, yellow, and green fruits and vegetables. Particularly good sources of beta-carotene include various greens (collard, turnip, spinach, lettuce), mangos, cantaloupe melons, peppers, pumpkin, carrots, and sweet potatoes (6). Beta-carotene, along with alpha-carotene and beta-cryptoxanthin, is a source of provitamin A. Once converted to vitamin A, health benefits derived from these compounds include maintenance of normal eye health, epithelial function, embryonic development, and immune system function (7).

Epidemiological studies have found that diets high in fruits and vegetables are associated with decreased cancer risk. These findings led to the belief that that beta-carotene may help reduce the risk of lung cancer (5). However, several human intervention studies in which beta-carotene supplements were used found that these supplements may increase the risk for lung cancer in high risk populations (smokers, asbestos-exposed workers) (8, 9). These results have led to questions as to whether beta-carotene is actually the active component responsible for the associations between fruit and vegetable consumption and cancer risk, or if other components or a combination of components is responsible for these effects (5). In addition to the debated anti-cancer benefits of beta-carotene, this carotenoid has also been proposed to decrease risk factors for cardiovascular disease.

2.1.2 Lycopene

Lycopene ($C_{40}H_{56}$) is a hydrophobic, acyclic carotenoid containing eleven conjugated double bonds (**Figure 2.1**) (10, 11). Tomatoes, watermelon, guava, and grapefruit are the main sources of lycopene in the diet (6). In these raw plant products, approximately 95% of lycopene is found in the all-trans form (11, 12) and is located in the photosynthetic pigment-protein complex of the thylakoid membrane (13). In some thermally processed foods and in human serum and tissues, higher quantities of cis isomers are found (12, 14).

The popularity of lycopene as a bioactive food component has often stemmed from a number of epidemiological studies that have concluded that diets rich in high lycopene foods are associated with a reduced risk of several diseases. In some cases, cell culture and dietary intervention studies have provided additional evidence that lycopene may play a positive role in health. Consumption of high lycopene foods has been proposed to reduce the risk of diseases including cardiovascular disease (10), and cancers of the prostate (1, 15, 16), breast (11), cervix, colon, esophagus, skin, pancreas, bladder and stomach (1, 12, 14). Of these conditions, the most evidence exists for lycopene and a reduced risk of prostate cancer

2.1.3 Lutein and zeaxanthin

Lutein and zeaxanthin ($C_{40}H_{56}O_2$) (**Figure 2.1**) are oxygenated carotenoids, making them members of the xanthophyll group of carotenoids (17). Lutein and zeaxanthin are stereo isomers, which complicates analytical techniques for determining the quantities of each stereo isomer present, and likewise, has created difficulties in

determining the influence of the individual isomers on human health (18, 19). Particularly good sources of these carotenoids are leafy greens like spinach, collard greens, and kale, corn, persimmons, and broccoli (6). Commercially produced lutein is derived from marigolds and is used in the poultry industry to impart yellow color to the yolks of eggs and the skin of broilers (20).

Some evidence suggests that lutein and zeaxanthin are associated with a reduced incidence of age-related macular degeneration and cataracts as determined by epidemiological and intervention studies (17, 21, 22). The mechanism by which these carotenoids are thought to decrease macular degeneration and cataract formation, is increasing macular pigments in the ocular tissues, which helps to filter damaging blue light, thus preventing oxidation damage that eventually leads to tissue damage (17, 23, 24). However, other studies have not found a relationship between lutein and zeaxanthin and eye health (21), indicating that more work is needed in this area of nutrition (23).

While eye health is the predominant health benefit associated with lutein and zeaxanthin, beneficial effects of these carotenoids have been proposed for other health conditions as well. Lutein has been proposed to reduce risk factors for coronary heart disease and stroke, breast cancer, and improving skin health, although research in these areas is still limited (17, 19).

2.1.4 Proposed physiological actions

A number of physiological actions have been proposed for carotenoids. These actions have been theorized based on a combination of studies on model systems as well as a current understanding of how carotenoid chemistry might inhibit disease progression.

There are still many questions over the exact chain of reactions responsible for these physiological actions as well as why certain carotenoids accumulate at high concentrations in some tissues of the body, but not others. More research is needed to elucidate these questions of tissue specificity and mechanistic action.

One of the mechanisms proposed for carotenoid protection of both plant and animal tissues is the quenching of singlet oxygen (25). Singlet oxygen is capable of damaging both DNA and lipids within cells (26). In organic solvents, several carotenoids, including those already discussed, have been found to have exceptional ability to quench singlet oxygen (26-28).

Carotenoids have also been found to act as free radical scavengers, reducing lipid oxidation reactions in certain model systems (29). Beta-carotene has been proposed to aid in the prevention of cardiovascular disease, possibly by protecting LDL cholesterol from oxidation or by reducing platelet aggregation (30), which would likely be due to the radical scavenging capacity of this compound.

Another mechanism by which carotenoids may help to reduce the risk of certain cancers is by participating in gap junctional communication. This allows for cell-to-cell communication, which, when functioning properly, helps to inhibit the growth of altered cells (31) that might otherwise generate tumors. Gap junctional communication has been found to be stimulated by beta-carotene (31) and has also been proposed as a mechanism of action for lycopene (25, 32).

2.1.5 Influence of isomeric form on bioavailability and bioactivity

Carotenoids are found in multiple isomeric forms due to the configuration of the long, conjugated system of double bonds characteristic of this group of pigments. The orientation of the double bonds is responsible for the shape and length of the molecule. There is some evidence that isomeric form may be an important factor in the bioactivity of certain carotenoids. For instance, *cis*-lycopene is found at high levels in human serum and tissues, while in most plant tissues and foods, the all-*trans* form predominates (33). Unlike lycopene, the *trans* isomer of lutein is the predominant form found both in raw plant materials and some human tissues, and has also been found to be preferentially absorbed by cell cultures (34).

The finding that *cis*-lycopene isomers concentrations are higher in animal tissues than in raw plant materials has created debate within the nutrition community as to whether *cis* isomers may be preferentially absorbed by the body or *trans*-lycopene is converted to *cis* once consumed (14, 32, 33, 35, 36). Bile acid micelles made from a mixture of lycopene isomers were found to have higher incorporation levels of *cis* isomers (33). In the same study, soybean oil with added lycopene was fed to ferrets and it was found that significantly higher levels of *cis*-lycopene isomers were present in lymph secretions than in stomach or intestinal contents, indicating that *cis* isomers were more bioavailable than all-*trans* isomers (33). Plasma lycopene content results found in a trial using human subjects fed sauce made from tangerine tomatoes that have high levels (97%) of *cis* isomers add additional support to the hypothesis that *cis* lycopene isomers are more bioavailable than all-*trans* isomers (36). Since all-*trans* lycopene is linear, it may be more likely to form into crystals that have lower solubility than its *cis*

counterparts. In addition, the linear configuration of the all-*trans* lycopene might have difficulty fitting into bile micelles compared to curved *cis* forms thus making it difficult for the all-*trans* lycopene to be transported from the food to the intestinal microvilli, which they can be absorbed into the blood. *Cis* isomers of beta-carotene have also been found to have greater efficiency of incorporation into micelles than all-*trans* beta-carotene *in vitro* (37, 38), but were not found to be preferentially absorbed by Caco-2 human intestinal cells (38). Difference in carotenoid solubility and shape may help explain their differences in bioavailability (33).

There is also evidence that thermal processing makes carotenoids more bioavailable. The reasoning behind this phenomenon is that the heating, increase in surface area, and agitation typically associated with thermal processing is likely to lead to breakdown of the cellular matrix of the plant material and may also induce *trans* to *cis* isomerization (14, 39-44).

2.2 Carotenoids as bioactive food ingredients

The evolving evidence that carotenoids can play a role in preventing disease has sparked interest in incorporating these compounds into functional foods. There is some evidence that carefully incorporated carotenoids may be more bioavailable than their natural sources. For instance, some carotenoids, like lycopene can form crystalline aggregates in chromoplasts of plants cells (13), which may lower their solubility and bioavalibility. Heat processing and presence of lipids in the food matrix has been shown to improve the bioavalibility and *trans*-*cis* isomerization of beta-carotene and lycopene-rich foods, as would be expected because carotenoids are lipophilic and heat can induce

isomerization (5, 14, 39-43, 43, 44, 44-46). Theoretically, it might be possible to manipulate a lycopene ingredient to optimize solubility, isomeric form and other attributes influencing bioavailability.

Incorporating bioactive carotenoids into foods that do not naturally contain high amounts of these compounds may offer great health benefits and provide new markets for food products. Addition of carotenoids to functional foods could be advantageous since they could be incorporated into an organic solution (i.e. an edible oil), which makes them more bioavailable than carotenoids in a plant cellular matrix. However, there are a number of stability issues that must be overcome before carotenoids can be successfully used as functional food ingredients since carotenoids placed in organic solutions, are less stable than carotenoids naturally occurring in tissues (2, 3). Therefore, it is essential to understand the mechanisms by which carotenoids may be degraded, in order to develop strategies for optimizing the stability of these bioactive ingredients.

2.3 Carotenoid oxidation

The conjugated polyene chain that is characteristic of carotenoids also makes these compounds susceptible to degradation from a number of agents. Depending on the carotenoid, the terminal end groups may also suffer degradation in certain environments (47). A number of carotenoid oxidizing agents have been identified. Carotenoid degradation pathways are highly influenced by the agent involved in the initiation of degradation. Common oxidation mechanisms are discussed below and summarized in **Figure 2.2**. Once oxidation has been initiated by one of a number of oxidizing agents, carotenoids may further react with themselves or other chemical species within the

environment to form a plethora of products. A summary of these products can be found in **Figure 2.3**. In a functional food fortified with carotenoids, oxidative damage could result in loss of both product quality (color loss, rancidity, etc.) and bioactivity. These detrimental aspects of carotenoid oxidation highlight how it is important to have a critical understanding of the mechanisms of carotenoid oxidation so that technologies can be developed to optimize stability in functional foods.

2.3.1 Autoxidation

Reaction of carotenoids with atmospheric oxygen has been found to occur with relative ease, especially in systems consisting of purified carotenoids in organic solvents. Autoxidation of beta-carotene in benzene or tetrachloromethane in the dark at 30°C under one atmosphere of oxygen or by bubbling oxygen through the solvent was found to occur with an induction period of less than one hour, followed by rapid production of oxidation products. Under these conditions, beta-carotene was completely consumed within 30 hours. A combination of HPLC, FT-IR, and GC-MS were used to monitor the reaction and identify over twenty oxidation products (see **Figure 2.3**) (48). Further information about the reaction occurring in this system was gathered by adding AIBN (2,2'-azo-*bis*-isobutyronitrile (a free radical reaction initiator), BHT (butylated hydroxytoluene), or alpha-tocopherol to the system. Both BHT and alpha-tocopherol reduced the rate of oxidation product production, while AIBN increased the rate of product formation. These results indicate that beta-carotene oxidation is likely to follow a free radical chain reaction. The authors of this study propose that autoxidation may be begin as beta-carotene in solution forms isomers via a biradical process (this may occur easily in

solvent at 30°C), as seen in **Figure 2.4**. The twisting of the molecule during the isomerization process may lead to an unpaired spin state, which can react easily with oxygen to form a carbon-peroxyl triplet biradicals. These may go on to form endo-peroxides or to react with a neutral beta-carotene molecule, forming an epoxide and a carotene alkoxyl radical. From the compounds detected in this study, it was concluded that the autoxidation process results first in the production of epoxides, carbonyl compounds, and an uncharacterized oligomers, followed by further oxidation reactions of these compounds to produce secondary short chain carbonyl compounds, carbon dioxide, and carboxylic acids. (48)

Beta-carotene adsorbed to a C_{18} solid phase also exhibited autoxidation when oxygen-saturated water was flowed continuously over the solid support. Using a combination of HPLC, UV-Vis spectroscopy, and electrospray LC-MS, a number of isomers and degradation products were detected. These included 13-*cis*, 9-*cis* and a di-*cis* isomers, beta-apo-13-carotenone, beta-apo-14'-carotenal, beta –carotene 5,8-epoxide, and beta-carotene 5,8-endo-peroxide (49). Retinoic acid, a compound derived from some provitamin A carotenoids (50), has been proposed to react directly with oxygen in the triplet state, at room temperature (51). This proposed pathway was based on the products formed when retinoic acid was exposed to 5-40 atm oxygen in 90% ethanol at room temperature. The products formed under these conditions were *trans*- and *cis*-5,8-epoxy-5,8-dihydroretinoic acid, 5,6-epoxy-5,6-dihydroretinoic acid, 5,8-epidioxy-5,8-dihydroretinoic acid, 2-methyl-6-oxo-2,4-heptadienal, beta-ionone, cyclocitral, dihydroactinidiolide, and 5-hydroxy-8-oxo-6,7-dihydroretinoic acid. 5,8-epidioxy-5,8-dihydroretinoic acid and 5-hydroxy-8-oxo-6,7-dihydroretinoic acid. These products were

formed in the presence of peroxyl radical scavenger (2,6-di-*tert*-butyl-4-methylphenol) even though the formation of all other products was greatly inhibited, suggesting that these are products of direct oxygen addition while the other products produced without BMP present are due to concurrent autoxidation. The research group postulated that the mechanism of oxygenation may involve the collapse of electron donor-acceptor complexes forming carbon-peroxyl biradicals followed by intersystem crossing and ring closure (51).

2.3.2 Thermal degradation

Thermal treatment to carotenoids in the presence of oxygen results in the formation of volatile compounds and larger non-volatile components (52). Kanasawud and Crouzet (53) proposed a sequence for beta-carotene degradation based on the products found during the heating of beta-carotene at 97°C for up to 3 hours in the presence of air as determined by GC-MS and absorption spectrophotometry. They suggested that beta-carotene reacts with oxygen to form 5,6-epoxy-beta-carotene, which can then convert to mutatochrome, 5,6,5',6'-diepoxy-beta-carotene, or luteochrome. Luteochrome may convert to aurochrome, which may then be cleaved to form dihydroactinidiolide. 2,5,6-epoxy-beta-carotene may cleave to form 5,6-epoxy-beta-ionone, which may be converted to beta-ionone, 2-hydroxy-2,6,6-trimethylcyclohexanone, 2,6,6-trimethylcyclohexanone, and 2-hydroxy-2,6,6-trimethylcyclohexane-1-carboxaldehyde. 2-Hydroxy-2,6,6-trimethylcyclohexane-1-carboxaldehyde can form beta-cyclocitral while 2-hydroxy-2,6,6-trimethylcyclohexanone can form 2,6,6-trimethyl-2-cyclohexen-1-one (53).

Marty and Berset (54), found that heating pure beta-carotene at 180ºC for two hours resulted in the formation of a number of *cis* isomers as well as oxidation products as determined by HPLC. This work also showed that the level of air circulation in the sample increased the degradation of beta-carotene because of the greater likelihood of beta-carotene and oxygen interaction. The resulting compounds found in this work led the researchers to conclude that all of the double bonds of beta-carotene could be oxidized and that the breakage of these bonds is likely to occur starting at the terminal end of the molecule and proceeding towards the center of the molecule.

At lower temperatures (60ºC), with a stream of oxygen being passed through beta-carotene in toluene, El-Tinay and Chichester (55), produced evidence that beta-carotene terminal double bonds could be broken, producing various epoxides as determined by chromatography followed by absorption spectra determination. This research showed that there was no lag phase in the decomposition of beta-carotene, ruling out autoxidation as a mechanism for degradation. They also showed that the reaction of oxygen with beta-carotene might be catalyzed by metals due to their findings that cupric stearate increased the rate of reaction 4.3 fold (55). This research suggests that metals may have also been involved in the other autooxidation and thermal oxidation experiments as well since metal reactivity was not controlled in these studies. Another study (56) of beta-carotene in toluene conducted at 60ºC with oxygen produced a complex mixture of products including, but not limited to epoxides as found by El-Tinay and Chichester. These products led the researchers to conclude that under these oxidation conditions, various radical species are formed and can react with oxygen to form peroxyl radicals, which can undergo propagation reactions with additional carotenoids. Even under vacuum, higher

temperature treatment (240°C) of crystalline beta-carotene resulted in the formation of toluene, m-xylene, p-xylene, ionene, and 2,6-dimethylnaphthalene as determined by gas-liquid chromatography and infrared, nuclear magnetic resonance, and mass spectroscopies (57)

2.3.3 Photodegradation

Light degrades carotenoids, and several mechanisms of action have been proposed. Photooxidation produces species thought to be carotenoid radical cations (58, 59). Laser flash photolysis studies have produced evidence to suggest that rapid bleaching of beta-carotene in some solvents like chloroform, can occur due to light exciting the beta-carotene molecules, which then instantly react with the solvent (chloroform in this case) to form either a carotenoid-solvent free radical adduct or a beta-carotene radical (due to hydrogen abstraction). The same work has also shown that the beta-carotene molecules in the excited state may return to ground state, where they may be attacked by radical by-products created during the above reaction and undergo a slower degradation process thought to possibly form beta-carotene radical cations (59).

2.3.4 Singlet oxygen

In a mechanism similar to carotenoid excitation described in photodegradation, light can also excite sensitizers like chlorophylls (60), leading to the formation of singlet oxygen ($^1O_2$). As seen in Equation 2.1, singlet oxygen may then react with neutral carotenoids to produce excited state carotenoids (^{3}Car*) . Once in an excited state, the carotenoid may return to ground state by releasing energy by vibrational and rotational

interactions with the surrounding solvent (Equation 2.2) (1, 60-62). The rate of singlet oxygen quenching has been found to be 17-31, 13-14, 12-12.6, and 6.64-11 x 10^9 M^{-1} s^{-1} for lycopene, beta-carotene, zeaxanthin, and lutein respectively (26, 27).

$$^1O_2 + Car \rightarrow {}^3O_2 + {}^3Car^* \quad (2.1)$$

$$^3Car^* \rightarrow Car + Heat \quad (2.2)$$

Additionally, a computational study using density functional theory has concluded that while the physical quenching pathway described in Equations 2.1 and 2.2 is the most favored mechanism for carotenoid and singlet oxygen interaction, the excited state carotenoids may also follow a chemical degradation pathway that is still not well understood (63). This study suggests that the chemical pathway might involve a direct attack on the double bonds of the carotenoid by singlet oxygen, which forms biradicals that can eventually lead to carbonyl chain cleavage products (63). Yamauchi et al., proposed two products resulting from the chemical oxidation of carotenoids by singlet oxygen. In their work, beta-carotene 5,8-endo-peroxide and beta-carotene 5,6-epoxide were produced during chlorophyll-sensitized photoxidation of beta-carotene in methyl linoleate (64) Of these two products, it has been proposed that beta-carotene 5,8-endo-peroxide is the primary product, while beta-carotene 5,6-epoxide results from the creation of an oxygen-centered radical during reaction with singlet oxygen, which subsequently abstracts a hydrogen from the lipid medium and undergoes an S_Hi reaction to form the final product. Oxidation of beta-carotene with singlet oxygen was also found to produced beta-apo-14'-carotenal, beta-apo-10'-carotenal, beta-apo-8'-carotenal, beta-ionone, and beta-carotene 5,8-endo-peroxide as determined by HPLC, GC-MS, and LCMS/MS (65). Chemical oxidation products were also found to form during the irradiation of lycopene

in the presence of oxygen and methylene blue (sensitizer). These products were determined to be apo-6'-lycopenal, and a mixture of seven short-chain oxygenated compounds including 2-methyl-2-hepten-6-one using MS, UV, IR, and ^{1}H- and ^{13}C-NMR spectra. The formation of these products led to the hypothesis that singlet oxygen, formed in the presence of the methylene blue sensitizer, reacted with lycopene to form a cyclic peroxide at C_5-C_6. Being a relatively unstable compound, this cyclic peroxide could easily undergo scission to form the detected products (66).

2.3.5 Acid

` Exposure to acids is thought to produce ion-pairs, which can then dissociate to form a carotenoid carbocation. This process can be seen in Equation 2.3 (67).

$$Car + AH \leftrightarrow (CarH^{+\cdots}A^{-}) \leftrightarrow CarH^{+} + A^{-} \quad (2.3)$$

Optical spectra of carotenoid carbocations have been reported for beta-carotene, 8'-apo-caroten-8'-al, and canthaxanthin when exposed to trifluoroacetic acid in benzene, CH_2Cl_2, and acetonitrile solutions (67). He and Kispert (68) showed that canthaxanthin and 8'-apo-beta-caroten-8'-al incorporated into sol-gels could be degraded by sulfuric acid (pH 3-3.5).

2.3.6 Iron and iodine

Some evidence indicates that iron may be capable of directly interacting with carotenoids to produce degradation products. In most work completed to date, ferric chloride is the commonly used iron oxidizing agent for studying the degradation of carotenoids (58, 68-76). Beta-carotene (68-70, 73-76), canthaxanthin (68-71, 73, 75, 76),

8’-apo-beta-caroten-8’-al (68, 70-72, 76), ethyl 8’-apo-beta-caroten-8’-oate (70, 72, 74, 76), 7’-apo-7’,7’-dicyano-beta-carotene (68), ethyl-6’-apo-beta-caroten-6’-oate (72), ethyl 4’-apo-beta-caroten-4’-oate(72), 6-apo-beta-caroten-6’-al (72), 4’-apo-beta-caroten-4’-al(72), 8’-apo-beta-carotene-8’-nitrile (72), 6’-apo-beta-carotene-6’-nitrile (72),7’c-cyano-7’-ethoxycarbonyl-7’-apo-beta-carotene (69), 7’,7’-dimethyl-7’-apo-beta-carotene (69), and 4’-apo-beta-carotene-4’-nitrile (72) have all been degraded by iron, in studies using carotenoids and ferric chloride in dichloromethane (69, 71-76) or in sol-gels containing iron in the aqueous fraction (68). The proposed interaction of neutral carotenoid with ferric iron can be seen in Equation 2.4.

$$Car + Fe^{3+} \leftrightarrow Car^{\bullet +} + Fe^{2+} \quad (2.4)$$

The reaction results in the formation of a carotenoid cation radical and ferrous iron as determined by comparison of the optical (69, 70, 72-74) and stop-flow absorption spectra (72) to the spectra produced from oxidation of carotenoids in cyclic voltammetry, bulk electrolysis (69, 71-73), EPR spectroscopy (70), or square voltammetry (72, 77) studies. This reaction can occur in the presence or absence of near-UV to visible light. However, irradiation with near-UV to visible light increases reaction rates (58, 73). When light is present, solutions of carotenoids (specifically beta-carotene and canthaxanthin) and ferric chloride in dichloromethane have regenerated ferric iron from the ferrous iron produced in Equation 2.4. The reproduced ferric iron can go on to react with remaining neutral carotenoid or may react with carotenoid cation radicals produced in Equation 2.4 to form dications as seen in Equation 2.5 (69, 73).

$$Car^{\bullet +} + Fe^{3+} \rightarrow Car^{2+} + Fe^{2+} \quad (2.5)$$

Gao and Kispert found evidence of dication formation by examining UV/vis spectra and Osteryoung square-wave voltammetry results after the addition of 4 equivalents of $FeCl_3$ to ethyl-all-*trans*-8'apo-beta-caroten-8'-oate or beta-carotene in dichloromethane (74).

The production of carotenoid dications may also be concentration dependent with respect to iron. Increasing levels of ferric chloride result first in formation of cation radicals (~0.5 equivalents ferric chloride). With additional ferric chloride (2 or more equivalents), dication carotenoids were formed in the presence of ferric chloride and beta-carotene, 7'-cyano-7'-ethoxycarbonyl-7'-apo-beta-carotene, canthaxanthin, or 7',7'-dimethyl-7'-apo-beta-carotene. The transformation to a cation or dication alters the optical absorption spectra of the carotenoid. For all carotenoids tested in this study, the λ_{max} for the three species increased in the order of neutral carotenoid < dication < cation radical (69).

Iodine is a milder oxidizing agent than ferric chloride (69), yet, it has been found to degrade 8'-apo-beta-caroten-8'-al, 7'-apo-7',7'-di-cyano-beta-carotene, ethyl 8'-apo-beta-caroten-8'-oate, canthaxanthin (70) and beta-carotene to cation radicals as determined by UV-vis and EPR spectroscopy (70, 78). Reaction of pure beta-carotene with ^{123}I in benzene and light petroleum resulted in beta-carotene-tri-iodide as determined by elemental analysis and Mössbauer spectroscopy. This product is formed by a one-electron transfer from beta-carotene to tri-iodide (79).

2.3.7 Radicals

In some systems, such as foods, radicals may already be present in the medium due to reactions such as lipid oxidation (62). Carotenoids have been found to exhibit both

antioxidant and prooxidant effects in systems containing pre-formed radicals depending on the type and level of carotenoid used, oxygen concentration present, and the polarity of the solvent (29). When radicals are present in a system, several mechanisms of carotenoid interaction are possible. These reactions include electron transfer, hydrogen abstraction, and addition of radical species to form carotenoid-radical adducts (see **Figure 2.2**) (2, 80, 81). The initial products of electron transfer, hydrogen abstraction, and adduct formation reactions, can in turn react with additional species via a number of mechanisms, including other radical interactions. A variety of products, summarized in **Figure 2.3**, result from these secondary reactions. Each of the three radical interaction mechanisms, and the subsequent reactions of the resulting products will be described below.

2.3.7.1 Electron transfer

Some studies suggest that neutral carotenoids are capable of participating in electron transfer reactions with radicals as well as with metals like iron as discussed previously. In these reactions, carotenoid radical cations are formed (Equation 2.6) (2, 73, 74, 80, 82, 83). Beta-carotene, canthaxanthin, zeaxanthin, astaxanthin, and lycopene have been shown to form radical cations by electron transfer reactions with tryptophan radical cations in a pulse radiolysis study using carotenoid containing micelles (82). Pulse radiolysis has also shown that radicals of nitrogen dioxide react with carotenoids in this manner in lycopene, lutein, zeaxanthin, astaxanthin or canthaxanthin in tert-butanol/water mixtures. The same reaction mixtures and pulse radiolysis techniques also found thiyl-sulphonyl radicals to produce carotenoid radical cations, but an

uncharacterized intermediate (possibly and ion-pair) was detected as well (83). The likelihood of electron transfer reactions taking place may depend on the type of carotenoid and radical. For instance, there is evidence from laser flash and steady-state photolysis studies that beta-carotene is not likely to interact with peroxyl radical in this manner and are more likely to undergo adduct formation or hydrogen abstraction reactions (discussed in sections 2.3.7.2 and 2.3.7.3) (84).

$$ROO\bullet + Car \rightarrow ROO^{-} + Car\bullet^{+} \quad (2.6)$$

If carotenoid radical cations are formed, a number of reactions may occur. One path they may follow is to interact with oxygen to form a carotenoid peroxyl radical cation (Equation 2.7), which might then be reduced by another carotenoid or ferrous iron, if present, to create a peroxide form of the carotenoid (Equations 2.8 and 2.9) (74). In one study, this series of reactions was determined to produce high amounts (~90% yield) of the 5,8-endo-peroxide of both all-*trans*-beta-carotenene and ethyl all-*trans*-8'-apo-beta-caroten-8'-oate as determined by APCI LC-MS and ^{1}H NMR(74). If iron is present, reactions previously covered in discussion of Equation 2.5, may occur. One study has also found that bulk electrolysis of canthaxanthin in CH_2Cl_2 with ferric chloride at low temperature (-10°C) followed by irradiation with UV-vis light for 1.5 minutes, can lead to the formation of carotenoid dimers. This is thought to occur by the formation of a radical cation that then reacts with neutral carotenoids to form dimers under these conditions (73). Pulse radiolysis studies have also found radical cations to decay in a bimolecular process, although the products were not determined (83)

$$Car\bullet^{+} + O_2 \rightarrow CarO_2\bullet^{+} \quad (2.7)$$

$$CarO_2\bullet^{+} + Fe^{2+} \rightarrow CarO_2 + Fe^{3+} \quad (2.8)$$

$$CarO_2\bullet^+ + Car \rightarrow CarO_2 + Car\bullet^+ \quad (2.9)$$

A final reaction series that carotenoid radical cations have been found to undergo in solvent systems is deprotonation. This reaction has been studied using canthaxanthin and beta-carotene in dichloromethane using electrochemistry and optical, MALDI-TOF, and EPR spectroscopy (75). The deprotonation reaction (Equation 2.10) was found to be enhanced by the presence of water in the solvent system (75). Density functional theory applied to this reaction has led to the conclusion that for beta-carotene radical cations, deprotonation at the 5 or 5' methyl group on the cyclohexene ring would result in the most stable product (77).

$$Car\bullet^+ \leftrightarrow \#Car\bullet + H^+ \quad (2.10)$$

$$\#Car\bullet + O_2 \rightarrow \#Car\text{-}OO\bullet \quad (2.11)$$

(# indicates one less proton)

It has been suggested that carotenoid radicals may be capable of reacting with oxygen to form additional radicals (Equation 2.11) (70). In addition, the deprotonated radical has been found to react with other deprotonated radicals to form dehydrodimers (Equation 2.12)(75).

$$\#Car\bullet + \#Car\bullet \rightarrow (\#Car)_2 \quad (2.12)$$

As already discussed, carotenoid dications may be formed by reaction of carotenoid radical cations with iron (Equation 2.5). Electrochemical oxidation of beta-carotene has also been shown to produce dications in tetrahydrofuran, dichloromethane and dichloroethane solutions as determined by EPR (85). Whether radical cations or dications are the predominant species formed, may depend on the structure of the carotenoid. Khaled et al. found that beta-carotene and beta-apo-8'-carotenal form

predominantly dications in experiments using simultaneous electrochemical-EPR of carotenoids in dichloromethane, while canthaxanthin produced predominantly radical cations (86).

If dications are produced, two additional decay reactions have been proposed. The dication may be able to react with a neutral carotenoid in the system, creating two radical cations (Equation 2.13) (87), or it may be deprotonated (Equation 2.14), in a pathway like that shown in Reaction 10, which is especially enhanced in the presence of water (69, 72, 73, 87). Reactions (2.13 and 2.14) were studied by Khaled et al. using EPR and cyclic voltametry (87). This work found evidence that dications of beta-carotene, canthaxanthin, and beta-apo-8'-carotenal in dichloromethane or dichloroethane can undergo both Reactions 2.13 and 2.14. A mixture of carotenoid radical cations and dications was produced using varied amounts of ferric chloride (1-2 equivalents) to oxidize canthaxanthin in dichloromethane, suggesting that Reaction 2.13 was occurring in this system (69).

$$Car^{2+} + Car \leftrightarrow 2\ Car^{\bullet +} \quad (2.13)$$

$$Car^{2+} \leftrightarrow \#Car^{+} + H^{+} \quad (2.14)$$

The deprotonation reaction (Equation 2.14) has been studied using electrochemistry (75), optical spectroscopy (73, 75), MALDI-TOF spectroscopy (75), and EPR spectroscopy (75), using canthaxanthin (73, 75) and beta-carotene (73, 75).

2.3.7.2 Hydrogen abstraction

Reaction of neutral carotenoid with a radical can result in the radical abstracting hydrogen. This results in the formation of a resonance stabilized carotenoid radical as

can be seen in Equations 2.15 and 2.16 (62, 88, 89). Liebler and McClure proposed this as a potential mechanism by which beta-carotene oxidation products were formed during oxidation with AMVN (2,4-dimethylvaleonitrile) radicals in benzene at 60°C based on atmospheric pressure chemical ionization mass spectrometry results (88). Woodall et al. also proposed Reaction 2.15 as a mechanism by which beta,beta-carotene might react with peroxyl radicals, postulating that the allylic C-4 position of the molecule might be the site of attack. This hypothesis was based on electron density calculations and UV-vis and mass spectrophotometry results produced during the reaction of beta,beta-carotene with AIBN radicals in the presence of methanol (89). Mortensen and Skibsted also proposed this mechanism as well as adduct formation as possible means by which alkyl, alkoxyl and alkylperoxyl radicals might react with beta-carotene (84).

$$ROO\bullet + Car \rightarrow ROOH + Car\bullet \quad (2.15)$$

$$OH\bullet + Car \rightarrow H_2O + Car\bullet \quad (2.16)$$

It has been proposed that the newly formed carotenoid radical might then encounter an additional alkoxyl or peroxyl radicals in the medium and react to form a non-radical product (Equations 2.17 and 2.18) (62, 88).

$$Car\bullet + RO\bullet \rightarrow Car\text{-}OR \quad (2.17)$$

$$Car\bullet + ROO\bullet \rightarrow Car\text{-}OOR \quad (2.18)$$

2.3.7.3 Adduct formation

Finally, radicals can also react with carotenoids to form radical adducts. This may occur with alkyl, alkoxyl, and peroxyl radicals as seen in Equations 2.19-2.21 (29, 81, 88). Laser flash and steady-state photolysis studies of beta-carotene indicate that at

least for this carotenoid, reaction with peroxyl radicals occurs much more slowly than with alkyl or alkoxyl radicals (84). Iannone et al. showed that beta-carotene and lutein can quench peroxyl radicals at low oxygen concentrations using EPR and spin trapping techniques (90) Laser flash photolysis studies of beta-carotene and canthaxanthin in aerated solutions of benzene with di-*tert*-butyl peroxide and toluene have provided evidence that the benzylperoxyl radical produced under these conditions reacts with the carotenoids to produce adducts that decay in a first-order reaction (81). Acetylperoxyl radicals produced during laser flash photolysis have also been shown to produce adducts with beta-carotene in aerated benzene at 20°C. The second order rate constant of this reaction is 9.2×10^8 $M^{-1}s^{-1}$ (91). Pulse radiolysis has also shown that glutathione thiyl radicals and 2-mercaptoethanol thiyl radicals react with carotenoids in a first-order reaction to produce adducts followed by bimolecular decay of the adducts in solutions of 10 μM lycopene, lutein, zeaxanthin, astaxanthin or canthaxanthin in *tert*-butanol/water mixtures (83).

$$\text{Car} + \text{R}\bullet \rightarrow (\text{Car-R})\bullet \quad (2.19)$$

$$\text{Car} + \text{RO}\bullet \rightarrow (\text{Car-RO})\bullet \quad (2.20)$$

$$\text{Car} + \text{ROO}\bullet \rightarrow (\text{Car-ROO})\bullet \quad (2.21)$$

Reaction with additional radicals is thought to result in addition products. Reaction 2.22 was proposed to be the mechanism by which beta-carotene addition products, detected by atmospheric pressure chemical ionization mass spectrometry, formed in reaction with AMVN-derived alkyl and alkoxyl radicals (88). The addition product in Equation 2.23 was suggested to be the main product of beta-carotene and lutein oxidation at low oxygen concentrations (90).

$$(\text{Car-RO})\bullet + \text{RO}\bullet \rightarrow \text{RO-Car-RO} \quad (2.22)$$

$$(\text{Car-ROO})\bullet + \text{ROO}\bullet \rightarrow \text{ROO-Car-ROO} \quad (2.23)$$

An additional pathway that radical adducts might follow involves reaction with oxygen. In what is thought to be a reversible reaction, oxygen may add to the radical adduct, producing a resonance stabilized, chain carrying peroxyl radical (Equation 2.24) (92). Liebler and McClure proposed this as well as hydrogen abstraction as potential mechanisms by which beta-carotene oxidation products were formed during oxidation with AMVN radicals in benzene at 60°C based on atmospheric pressure chemical ionization mass spectrometry results (88). El-Agamey and McGarvey were the first to observe reversible oxygen addition to neutral 7,7'-dihydro-beta-carotene radicals and neutral beta-carotene radicals utilizing phenylthiyl radicals produced by laser flash photolysis. The rate constants determined for the addition of oxygen to these neutral radicals were determined to be 0.64×10^4 $M^{-1}s^{-1}$ for beta-carotene radicals and 4.3×10^4 $M^{-1}s^{-1}$ for 7,7'-dihydro-beta-carotene radicals (93).

$$(\text{Car-ROO})\bullet + O_2 \leftrightarrow \text{ROO-Car-OO}\bullet \quad (2.24)$$

As first described by Burton and Ingold (92), the reaction described in Equation 2.24 is likely governed by oxygen pressure. In experiments with beta-carotene, rates of carotenoid autoxidation were found to decrease when oxygen levels were lowered . Similarly, as oxygen levels were lowered , beta-carotene had a greater antioxidant effect in the AIBN-initiated oxidation of tetralin and methyl linoleate in chlorobenzene at 30°C. These results led Burton and Ingold to conclude that as oxygen is lowered (often to levels similar to those in animal tissues), Reaction 2.24 shifts to the left and production of peroxyl radicals is reduced. At higher oxygen pressures, however, the reaction is driven

to the right and autoxidation rates increase due to hydrogen abstraction reactions like that shown in Equation 2.25.

$$ROO\text{-}Car\text{-}OO\bullet + RH \rightarrow ROO\text{-}Car\text{-}OOH + R\bullet \quad (2.25)$$

Peroxyl radical adducts, however, are also likely to follow other pathways. One path these radical adducts might follow is decay to carotenoid epoxides and new alkoxyl radicals (Equation 2.26), which may further the extent of oxidative degradation as the newly formed radicals attack other oxidizable substrates in the system (88). Mortensen found acetylperoxyl-beta-carotene radical adducts to decay with a first order rate constant of $1.35 \times 10^3\ s^{-1}$, and hypothesized that the decay of the adduct may follow this path (91). Yamauchi et al. found additional evidence for this type of pathway in the reaction products formed by AMVN-initiated peroxidation of beta-carotene in methyl linoleate or benzene. The products formed in the benzene study (as determined by HPLC followed by UV, infrared, ^1H and ^{13}C NMR and mass spectrometry) were 12-formyl-11-nor-beta, beta-carotene, 15'-formyl-15-nor-beta, beta-carotene, 19-oxomethyl-10-nor-beta, beta-carotene, 5,6-epoxy-5,6-dihydro-beta, beta-carotene, 13,15'-epoxyvinyleno-13,15'-dihydro-beta, beta-carotene, stereoisomers of 15',13-epoxyvinyleno-13,15'-dihydro-beta, beta-carotene, and 11,15'-cyclo-12,15-epoxy-11,12,15,15'-tetrahydro-beta,beta-carotene (94). In the methyl linoleate study, products formed (as determined by HPLC) included beta-carotene 5,6-epoxide, 13,15'-epoxyvinyleno-13,15'-dihydro-14,15-dinor –beta,beta-carotene, 15',13-epoxyvinyleno-13,15'-dihydro14,15-dinor-beta,beta-carotene, 11,15'-dihydrooxepin-beta-beta-carotene, 12-formyl-11-nor-beta, beta-carotene, and 15'-formyl-15-nor-beta, beta-carotene (64).

$$(Car\text{-}ROO)\bullet \rightarrow \text{Carotenoid Epoxide} + RO\bullet \quad (2.26)$$

2.4 Isomerization

Electron transfer reactions are also thought to play a role in isomerization of carotenoids. Some studies suggest that isomerization is mediated by carotenoid radical cations and possibly dications (Equations 2.27 and 2.28) formed from electron transfer from neutral carotenoids to radicals or compounds like iron that can easily transfer charge (71, 74, 95). This is thought to occur because of lower energy barriers for configural transformation in the cation species compared to those of neutral carotenoids (71, 95). Wei et al. 1997 (71) found that oxidation of canthaxanthin and 8'-apo-beta-caroten-8'-al resulted first in the formation of radical cations (as determined by optical spectroscopy), followed by formation of *cis*-isomers (as determined by HPLC). Canthaxanthin and beta-carotene in dichloromethane were also found to undergo this process as determined by bulk electrolysis with simultaneous absorption spectroscopy (95).

$$\textit{trans}\text{-Car}^{\bullet+} \leftrightarrow \textit{cis}\text{-Car}^{\bullet+} \quad (2.27)$$

$$\textit{trans}\text{-Car}^{2+} \leftrightarrow \textit{cis}\text{-Car}^{2+} \quad (2.28)$$

The *cis*-radical cations and dications are then thought to undergo reactions with neutral *trans*-carotenoids still present in the system to form either neutral *cis*-carotenoid or *cis*-radical cations as well as new *trans*-radical cations that can be recycled through the isomerization reaction sequence (Equations 2.29 and 2.30) (71, 95).

$$\textit{cis}\text{-Car}^{\bullet+} + \textit{trans}\text{-Car} \leftrightarrow \textit{cis}\text{-Car} + \textit{trans}\text{-Car}^{\bullet+} \quad (2.29)$$

$$\textit{cis}\text{-Car}^{2+} + \textit{trans}\text{-Car} \leftrightarrow \textit{cis}\text{-Car}^{\bullet+} + \textit{trans}\text{-Car}^{\bullet+} \quad (2.30)$$

Isomerization has also been found to occur during the oxidation of ethyl 8'-apo-caroten-8'-oate and beta-carotene by ferric chloride. This study proposed that if iron is present in the system, ferrous iron can react with the *cis*-radical cations or dications and

be oxidized to ferric iron and either the neutral *cis*-carotenoid or the *cis*-radical cation, just as seen in reaction with neutral *trans*-carotenoid in Equations 2.29 and 2.30 (74).

Isomerization is also thought to occur if carotenoids are exposed to acids. Similar to the carotenoid radical cation formed in electron transfer based isomerization, a carotenoid carbocation ($CarH^+$) is believed to be an intermediate in *trans-cis* isomerization. This carbocation has been predicted by Austin Model 1 (AM1) calculations of rotation barriers to have a lower barrier to rotation than neutral carotenoids, facilitating the isomerization process (67).

2.5 Emulsion delivery systems for bioactives

Carotenoids are primarily lipid-soluble, therefore one way to incorporate them into foods is in the oil phase of oil-in-water emulsions. Several bioactive food components such as omega-3 fatty acids have been incorporated into foods using emulsions as delivery systems. Emulsions have provided a stable delivery system for omega-3 fatty acids in ice cream and yogurt, with little consumer detection of altered sensory attributes (96-99). Emulsion-based bioactive delivery systems have several advantages since they are easy to incorporate into aqueous based products and they can retain their antioxidant properties once diluted into the food. An additional advantage of using emulsions as a delivery system for lipid-soluble bioactives, is that the emulsion system can contain high amounts of bioactives without having high total lipid concentrations thereby not requiring large quantities of total lipids to be added to the foods that would possibly be seen as undesirable by health-conscious consumers attempting to limit fat in their diet.

2.5.1 Engineering emulsions for oxidative stability

Given the variety of mechanisms by which carotenoids may be degraded, it is essential that systems for delivering these bioactive compounds into functional foods be carefully chosen. Emulsions may be particularly effective delivery systems for carotenoids because these systems can be engineered with numerous antioxidative functions (96). An emulsion is comprised of immiscible liquids, which are mixed to form a thermodynamically unstable system of droplets of one liquid (dispersed phase) surrounded by the second liquid (continuous phase) (100). In an oil-in-water emulsion, the dispersed phase would consist of oil droplets while water and water-soluble components would make up the continuous phase.

Emulsions are thermodynamically unstable because of positive free energy needed to increase the surface area between the two phases as well as density differences between the phases (101). To confer greater stability, emulsifiers are commonly added prior to homogenization. Emulsifiers are amphiphilic molecules with polar regions that orient towards the aqueous phase and non-polar regions that orient towards the oil phase of the emulsion. As droplets are formed during homogenization, the surface active emulsifiers adsorb to the oil/water interface, providing increased stability to the system by reducing the surface tension between the two phases. Small molecule surfactants are very effective because they rapidly absorb to the interface of newly formed lipid droplets and because their high surface activity maintains their association with the lipid droplet during food processing operations (101).

In emulsions, the region between the two immiscible phases, containing the emulsifier, is referred to as the interfacial region. Therefore emulsions consist of aqueous,

lipid and interfacial regions. All three regions of an oil-in-water emulsion may be manipulated to confer added stability to oil-phase components such as lipid-soluble bioactives. Much of the work to date in this area has focused on the manipulation of emulsions droplet interfaces to reduce lipid oxidation in oil-in-water emulsions.

2.5.2 Lipid oxidation in oil-in-water emulsions

The onset of lipid oxidation in oil-in-water emulsions is thought to be predominantly due to the breakdown of lipid hydroperoxides naturally present in the oil phase or the surfactants used to stabilize the emulsion (102, 103). These hydroperoxides can migrate to the interfacial region because they are more polar than unoxidized oils (104). At the emulsion droplet interface, hydroperoxides can interact with prooxidants in the aqueous phase, such as transition metals, forming highly reactive peroxyl and alkoxyl radicals (Equations 2.31 and 2.32) (102, 105). It is important to note that ferrous iron (Fe^{2+}) is much more reactive than ferric iron (Fe^{3+}), resulting in Reaction 2.32 proceeding at a rate 10^7 times faster than Reaction 2.32 (106).

$$Fe^{3+} + ROOH \rightarrow Fe^{2+} + ROO\bullet + H^{+} \quad (2.31)$$

$$Fe^{2+} + ROOH \rightarrow Fe^{3+} + RO\bullet + OH^{-} \quad (2.32)$$

The peroxyl and alkoxyl radicals formed can then react with unsaturated lipids (LH) within the oil droplets, forming alkyl lipid radicals (Equations 2.33 and 2.34), which in turn react with oxygen to form peroxyl radicals (Equation 2.35). The peroxyl radicals can then attack other unsaturated lipids, propagating lipid oxidation (Equation 2.36). Through a separate set of reactions, alkoxyl radicals can undergo β-scission reactions leading to fatty acid decomposition into aldehydes, ketones, and alcohols. Finally, the

radicals can react with one another forming non-radical products, in what are known as termination reactions (Equation 2.37). (105)

$$ROO\bullet + LH \rightarrow ROOH + L\bullet \quad (2.33)$$

$$RO\bullet + LH \rightarrow ROH + L\bullet \quad (2.34)$$

$$L\bullet + O_2 \rightarrow LOO\bullet \quad (2.35)$$

$$LOO\bullet + LH \rightarrow LOOH + L\bullet \quad (2.36)$$

$$LOO\bullet + LOO\bullet \rightarrow \text{nonradical products} \quad (2.37)$$

Lipid oxidation is often observed to occur in two phases. The first phase, the lag phase, has a low or undetectable level of oxidation products produced. This phase is followed by the exponential phase. During the exponential phase, concentrations of oxidation products increase dramatically and the characteristic odors and flavors associated with rancidity of food products are produced (107). During these phases, lipid oxidation can be monitored by measuring primary and secondary products. In the initial phase of oxidation, lipid hydroperoxides are formed and are often used as an indicator of the extent of primary oxidation. As oxidation progresses, the lipid hydroperoxides are decomposed to produce a variety of secondary oxidation products. Some of these products are volatile and can be monitored by gas chromatography.

2.5.3 Manipulation of emulsion components to reduce oxidation

Several strategies have been successfully employed to reduce the onset and rate of lipid oxidation reactions in oil-in-water emulsions. The components of the aqueous, oil, and interfacial regions can all play a role in altering the oxidation process, and several of

these strategies may also have potential in reducing the oxidation of emulsified carotenoids.

2.5.3.1 Aqueous phase manipulation

The aqueous or continuous phase of oil-in-water emulsions contains iron and other transition metals that may interact with lipids or other bioactive components in the oil droplets to produce the radical reactions like those described above. One way of reducing the likelihood of iron reacting with lipid hydroperoxides or carotenoids may be to alter the pH of the aqueous phase of the emulsion. Iron is more soluble at low pH, which may increase its ability to migrate to and interact with lipid droplets (108). In the oxidation of octadecane and methyl linolenate oil-in-water emulsions, oxidation was found to occur more rapidly at pH 3 than at pH 7, which is thought to be due to the greater solubility and reactivity of iron at pH 3 (109). Altering pH in bioactive delivery systems could be one possible method of inhibiting oxidation. However, this is often not possible because it could change other aspects of the food such as sensory characteristics.

Addition of compounds that can chelate metals, rendering them inactive, is another strategy to control oxidative degradation. EDTA (ethylenediamine tetraacetic acid), phosphates, citric acid, amino acids, proteins, and some polyssacarrides are capable of metal chelation (105, 110). EDTA has been shown to inhibit the oxidation of omega-3 oil-in-water emulsions stabilized by small molecule surfactants (111-113, 113). Proteins such as casein, whey and soy protein isolate can also chelate metals (97, 114, 115). Another chelator, citric acid, has been found to decrease oxidation in a model emulsion with an oil phase composed of menhaden oil-caprylic acid structured lipid (110) but citric

acid was not found to inhibit oxidation in menhaden oil-in-water emulsions stabilized by whey protein isolate (97). Chelators can inhibit oxidation in emulsions by reducing the reactivity of the metals or by promoting the transfer of iron from the lipid droplet to the aqueous phase of emulsions to decrease interactions between metals and oxidizable lipids (111, 116).

Amino acids, proteins and a variety of other natural and synthetic antioxidants may act as free radical scavenging antioxidants, delaying the initiation or propagation reactions of lipid oxidation. These antioxidants convert radicals to less reactive forms by donating a hydrogen atom to the radical (Equation 2.38). The most effective antioxidants will form a product that is a low energy antioxidant radical that is less reactive than the lipid radical and will not produce further lipid oxidation. (105, 117)

$$LOO\bullet + AH \rightarrow LOOH + A\bullet \quad (2.38)$$

Aqueous phase whey proteins have been found to reduce oxidation in salmon oil-in-water emulsions (118) and menhaden oil-in-water emulsions (119). Aqueous phase caseins and casein peptides have also been shown to reduce oxidation in Brij-stabilized corn oil-in-water emulsions (120). The ability of aqueous phase proteins to inhibit lipid oxidation in oil-in-water emulsions is likely due to a combination of both metal chelation and free radical scavenging. In beta-lactoglobulin, the amino acids cysteine, tryptophan and methionine all oxidized prior to unsaturated fatty acids suggesting that they are effective free radical scavengers in oil-in-water emulsions (121, 122). Transferrin, a protein that specifically binds iron, also inhibits oxidation in salmon oil-in-water emulsions, suggesting that metal chelation by proteins is also an important antioxidant mechanism (111).

A variety of other compounds may also inhibit lipid oxidation in oil-in-water emulsions. For instance, sugars may increase the viscosity of the aqueous phase and slow the rate of oxidation by increasing the time for prooxidants to reach the lipid droplet interface where oxidation reactions take place. Salt in certain concentrations may screen electrostatic interactions at the droplet interface that would attract iron to this site. Excess surfactant not adsorbed to the oil-water interface may also impact oxidation by forming micelles that may partition lipids, antioxidants, and prooxidants, and thereby alter their participation in oxidation reactions. (105)

2.5.3.2 Oil phase manipulation

The composition of the oil phase is an important factor in protecting lipid-soluble bioactives. In oxidative reactions, both the unsaturated fatty acids and bioactive lipids can be directly oxidized by reactive species like transition metals. In addition, radicals produced during the oxidation of one component may co-oxidize the other component. For example, an unsaturated fatty acid could oxidize first and produce free radicals that oxidize the bioactive lipids. Therefore, understanding the oxidative stability of the different components of the lipid phase is important for characterizing the overall stability of an emulsion delivery system.

The rate of lipid oxidation is dependent on the structural properties of the lipids. For instance, unsaturated fatty acids oxidize faster than saturated fatty acids due to the presence of double bonds. As the level of unsaturation of fatty acids increases, the time required for oxidation to proceed decreases since additional double bonds will make hydrogen abstraction easier and will provide additional sites for free radical attack (123).

Other bioactive lipids would also be expected to have differences in oxidative susceptibility. Both computational and experimental studies have produced evidence that some carotenoids are more susceptible to oxidation than others due to their structure. For instance, these studies have found lycopene to be less stable than beta- carotene due to its longer effective backbone of planar conjugated double bonds. These studies have also found zeaxanthin, lutein, and canthaxanthin to have greater stability than beta-carotene or lycopene due to hydroxyl and carbonyl end groups (124-126). Different phytosterol compounds, bioactives shown to reduce plasma cholesterol, have been proposed to have varied oxidative stability in emulsions based on how their structure determines surface activity at the oil-water interface (127).

The oil phase may also contain added or naturally occurring minor components that impact oxidative stability. Synthetic and natural free radical scavengers like tert-butylhydroquinone (TBHQ), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tocopherols added to oils can react with free radicals and decrease their ability to participate in oxidative reactions. Common vegetable oils that would likely be used for the production of bioactive delivery systems naturally contain minor amounts (1-2% of refined oil) of carotenoids, tocopherols/tocotrienols, chlorophyll, sterols, vitamins A and D and products of triacylglycerol hydrolysis (e.g., mono and diacylglycerols and free fatty acids) (128), which can also impact oxidation reactions. Of these naturally occurring compounds, some, like chlorophyll and free fatty acids, may have a prooxidant effect, but others may be protective. Tocopherols/tocotrienols and carotenoids are often considered to serve as antioxidants in oils, and may enhance the oxidative stability of the lipid carrier, the bioactive, or both in emulsion delivery systems. Understanding the role

of these minor lipid components on oxidative stability of mixture of lipid carriers and bioactive compounds will be important in designing effective bioactive lipid delivery systems.

Some work has examined the activity of minor oil components on the stability of oil as well as the minor components themselves. In one study, beta-carotene was found to suppress the rate of hydroperoxides formation in AMVN-initiated oxidation of methyl linoleate, but was not able to increase the lag phase of oxidation. When oxidation of this system was not initiated by AMVN, no suppression lipid hydroperoxide formation was found suggesting that beta-carotene primarily inactivated peroxyl radicals generated by AMVN. In this same model system alpha-tocopherol, acting as a free radical scavenger, prevented the degradation of beta-carotene by AMVN-initiated radicals until almost all the alpha-tocopherol was consumed (64). Another study investigated the degradation of beta-carotene and lutein in stripped corn oil as well as the effect of carotenoids on the promotion of lipid oxidation. At an incorporation concentration of 5 ppm in stripped oil, corn oil oxidation was decreased compared to carotenoid-free controls. However, at higher dosage levels of 10 – 30 ppm beta-carotene in oil, lipid oxidation was promoted, suggesting that concentration plays an important role in the stability of these systems. Lutein addition to the oil at all three testing levels showed a prooxidant effect. When liquid paraffin (a more stable medium) was used in place of corn oil, degradation of both beta-carotene and lutein were slower than in the corn oil. These results suggest that when combined, oils and carotenoids may both experience faster oxidation under certain conditions than they would alone. However, this study also showed that incorporation of both alpha-tocopherol and beta-carotene into the corn oil reduced lipid and beta-carotene

oxidation to a greater extent than alpha-tocopherol alone, indicating a synergistic effect between alpha-tocopherol and beta-carotene in reducing lipid oxidation as well as increasing the stability of the carotenoid (129).

2.5.3.3 Interfacial region manipulation

A body of recent work has shown that manipulation of both the charge and the thickness of the interfacial region can be useful in retarding lipid oxidation in emulsions. Surfactants can impart negative, neutral, or positive charges to emulsion droplets due to both the chemical structure of the surfactant and the pH of the aqueous phase. The net charge of emulsion droplets can be monitored by examining the zeta potential of the emulsion (112).

Generally, it has been found that positively charged oil-in-water emulsion droplets stabilized with cationic surfactants, are more oxidatively stable than negatively charged droplets formed using anionic surfactants. Several of the studies that have examined this phenomenon have used small molecule surfactants with known positive, neutral, or negative charges. One study examined differences in oxidation of corn oil-in-water emulsions stabilized by sodium dodecyl sulfate (SDS; anionic), Brij 35 (slightly anionic), or dodecyltrimethylammonium bromide (DTAB; cationic). At pH 6.5, oxidation was found to proceed fastest in the highly anionic, SDS emulsion, and the least amount of oxidation was found in the cationic, DTAB emulsion (104). Similar results were found in the study of oxidation in emulsions with an oil phase of octadecane and methyl linolenate or salmon oil using SDS, DTAB, and Tween 20 (a slightly anionic surfactant) (109, 111).

Surfactant proteins can also be manipulated to produce both positively and negatively charged droplets depending on whether the surrounding medium is above or below the pI of the protein. When proteins are at pH levels above the pI of the protein, droplets are negatively charged. As the pH approaches the pI, droplets become neutral in charge, and as the pH is lowered below the pI, proteins become cationic. Whey protein isolate stabilized menhaden oil emulsions (130), salmon oil emulsions (131), and canola oil/caprylic acid structured lipid (132) have all been shown to have greater oxidative stabilities when the pH of the emulsion is below the pI of the protein, creating positively charged droplets.

The lower oxidation in emulsions stabilized by cationic emulsifiers may be due to coulombic repulsive forces between the positive droplet surface charges and positively charged transition metals in the aqueous phase. Since the transition metals are repelled from the surface, they are less likely to interact with lipid hydroperoxides in the oil droplets, thus reducing the number of reactions that can lead to generation of free radicals (see Reactions 31 and 32) (104). Negatively charged surfactants attract positively charged transition metals. SDS has been shown to have a high association with both ferrous and ferric iron from the aqueous phase, and also a higher oxidation rate as a result of the iron breaking down pre-formed lipid hydroperoxides (112).

The thickness of the interfacial region is also thought to provide additional means for engineering emulsions for oxidative stability. Experiments using Brij 700 and Brij 76, showed that Brij 700 emulsions had greater oxidative stability. The only difference between these two surfactants is the size of the head group. Brij 700 has a head group 10 times larger than the polyoxyethylene groups than Brij 76, suggesting that the larger head

groups produced a thicker interfacial layer that acted as a physical barrier that restricted contact between transition metals and lipids (133). Differences in the interfacial thickness created by casein (~10 nm interfacial thickness) versus whey protein isolate (1-2 nm interfacial thickness) were also postulated to be a reason for slower oxidation in casein-stabilized droplets compared to whey protein isolate-stabilized emulsions (134)

2.5.4 Carotenoids in emulsion systems

A limited number of studies have been conducted incorporating carotenoids into the oil phase of oil-in-water emulsions. One of these studies used beta-carotene in linoleic acid stabilized by chondroitin sulfate sodium salt. This study examined the impact of light and *rac* alpha-tocopherol on the oxidation of these emulsions with varied amounts of beta-carotene . Oxidation was found to decrease with higher carotenoid content (10-100 ppm) when incubation was carried out in the dark at 30°C. However, when the same samples were incubated under light (4000 lux), oxidation was found to increase with higher concentrations of beta-carotene. Superoxide anions and hydrogen peroxides were found to form in the samples exposed to light and it was concluded that these species were responsible for the increased oxidation of light-exposed samples, though these species could be scavenged in the presence of tryptophan or mannitol. This work also showed that adding increasing concentrations of *rac* alpha-tocopherol (1-25 ppm) to a 25 ppm beta-carotene emulsion created a synergistic antioxidant effect in the dark, but had no effect in samples exposed to light. (135). Beta-carotene and alpha-tocopherol have also been tested in emulsions of canola oil/caprylic acid structured lipids-in-water stabilized by either whey protein isolate or sucrose fatty acid esters.

Beta-carotene incorporation at 200 ppm of the oil or 100 ppm combined with 100 ppm alpha-tocopherol showed a prooxidant effect compared to control emulsions in the formation of both primary (peroxide value) and secondary (anisidine value) oxidation products. (136)

Kiokias and Oreopoulou (137) studied annatto, paprika, marigold, and tomato extracts in tocopherol-stripped sunflower oil emulsions stabilized by Tween 20. The radical initiator, 2,2'-azobis-2-methyl-propanimidamide (AAPH), was added to the emulsion systems and oxidative stability was monitored using conjugated diene formation and solid phase microextraction (SPME) of headspace volitiles quantified by gas chromatography. All extracts added to the oil phase (active concentration of carotenoid was 1000 ppm in oil) were found to inhibit production of AAPH oxidation products (conjugated dienes and volatile aldehydes) compared to control emulsions at 60°C. The antioxidant nature of the carotenoids in this system is thought to be enhanced by the fact that AAPH rapidly combines with oxygen, resulting in a reduced oxygen environment , in which carotenoids and their oxidation products are less likely to form chain-carrying radical products. Antioxidant activity was highest in extracts containing high concentrations bixin and norbixin followed by extracts high in lutein, with high beta-carotene and lycopene extracts having the lowest activity. The same ordering of activity was found for the fastest carotenoid degradation as monitored by loss of absorbance at the maximum absorption wavelength for each carotenoid. Increasing the concentration of carotenoids in the oil phase from 500 to 5000 ppm showed large increases in antioxidant activity up to 1000 ppm for beta-carotene and bixin, and 3000 ppm for lutein. Combinations of 1000 ppm carotenoid extract and 0.1 or 0.2 mM of

either alpha or delta tocopherol was found to increase antioxidant activity with the exception of high-bixin extracts. Addition of 100 ppm of ascorbic acid showed a tendency (though non-significant) towards increased antioxidant activity as well.

Ribeiro and Schubert (138) performed studies of lycopene in a 20 wt % triglyceride oil emulsion (700 ppm lycopene in emulsion) stabilized by Tween 20, citric acid esters of mono- and diglycerides of fatty acids, or enzymatic hydrolisated soya lecithin with lysophospholipids. This emulsion was then dispersed into water, skim milk, or orange juice (10 ppm) and lycopene degradation was monitored. Lycopene was found to be relatively stable in orange juice over the 3 week testing period, while less stable in water and milk. In milk, approximately 45% of the lycopene had degraded within one week, while in water, approximately 80% of the lycopene was lost within this time. The three emulsifiers tested showed only slight differences in the stability lycopene. The lycopene emulsion was also tested using different means of removing oxygen from the system. Flushing the emulsion with nitrogen was found to actually increase the rate of oxidation, while incorporation of glucose oxidase led to much greater stability (though degradation still occurred). Addition of alpha-tocopherol at 1 to 10 ppm increased the stability of lycopene in all three media tested.

The scarcity of work on the topic of carotenoids in emulsion systems leaves much to be determined in this area of study. By carefully considering the mechanisms by which carotenoids degrade and by engineering emulsion systems to decrease interactions between carotenoids and prooxidant, it may be possible to produce a carotenoid delivery system with adequate stability to be incorporated into functional food products.

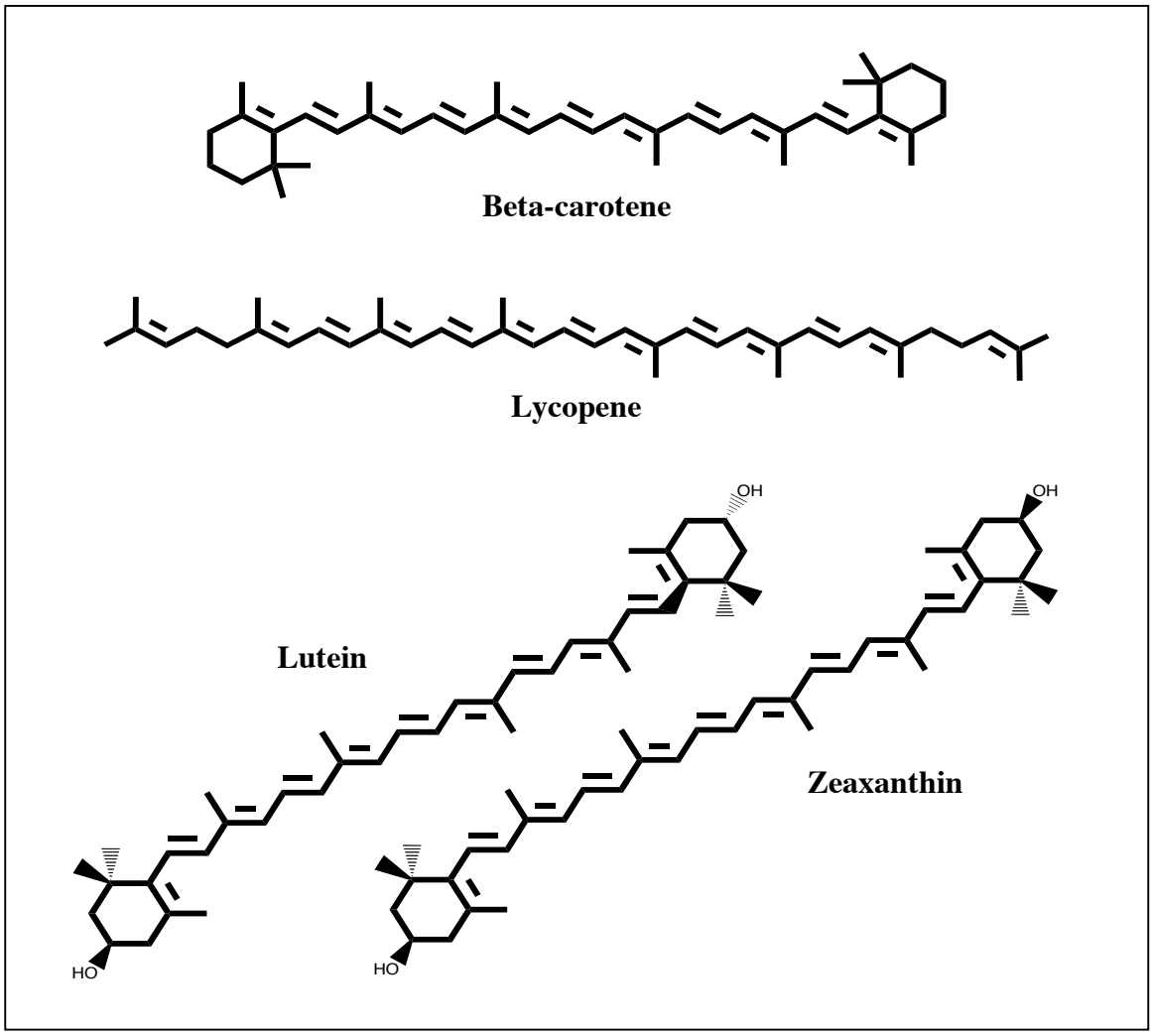

Figure 2.1: Structures of major carotenoids

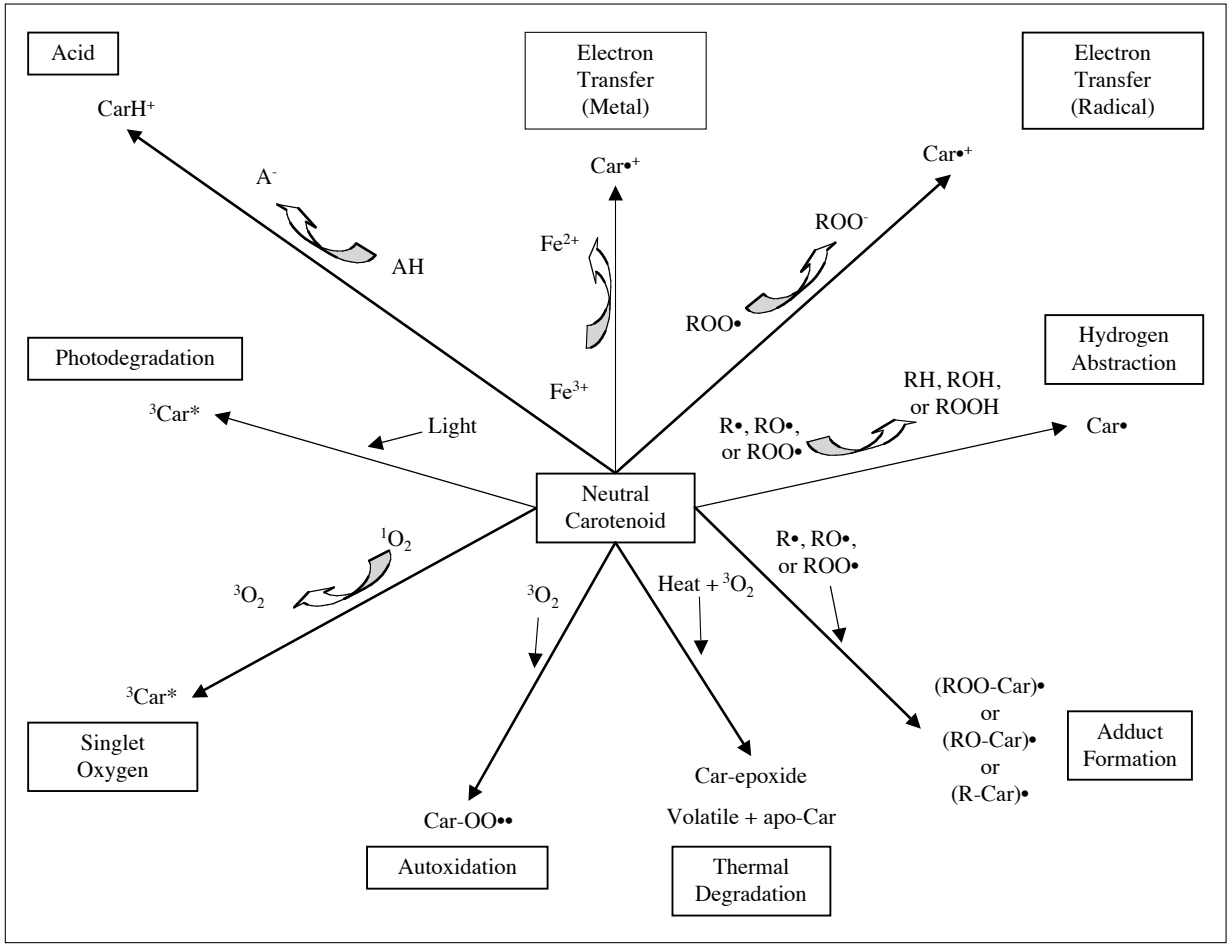

Figure 2.2: Mechanisms of carotenoid oxidation and initial products

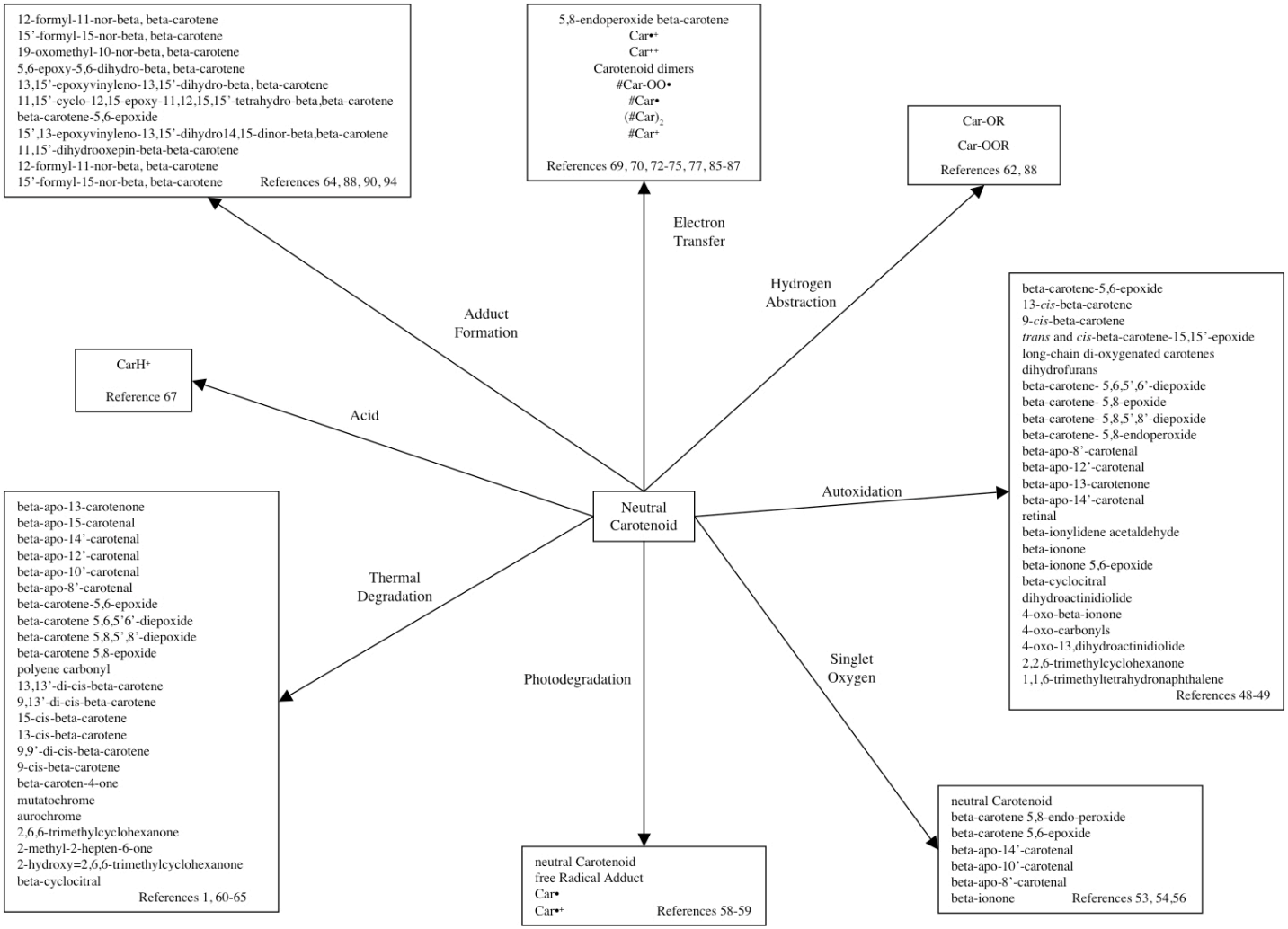

Figure 2.3: Overall oxidation products of beta-carotene oxidation

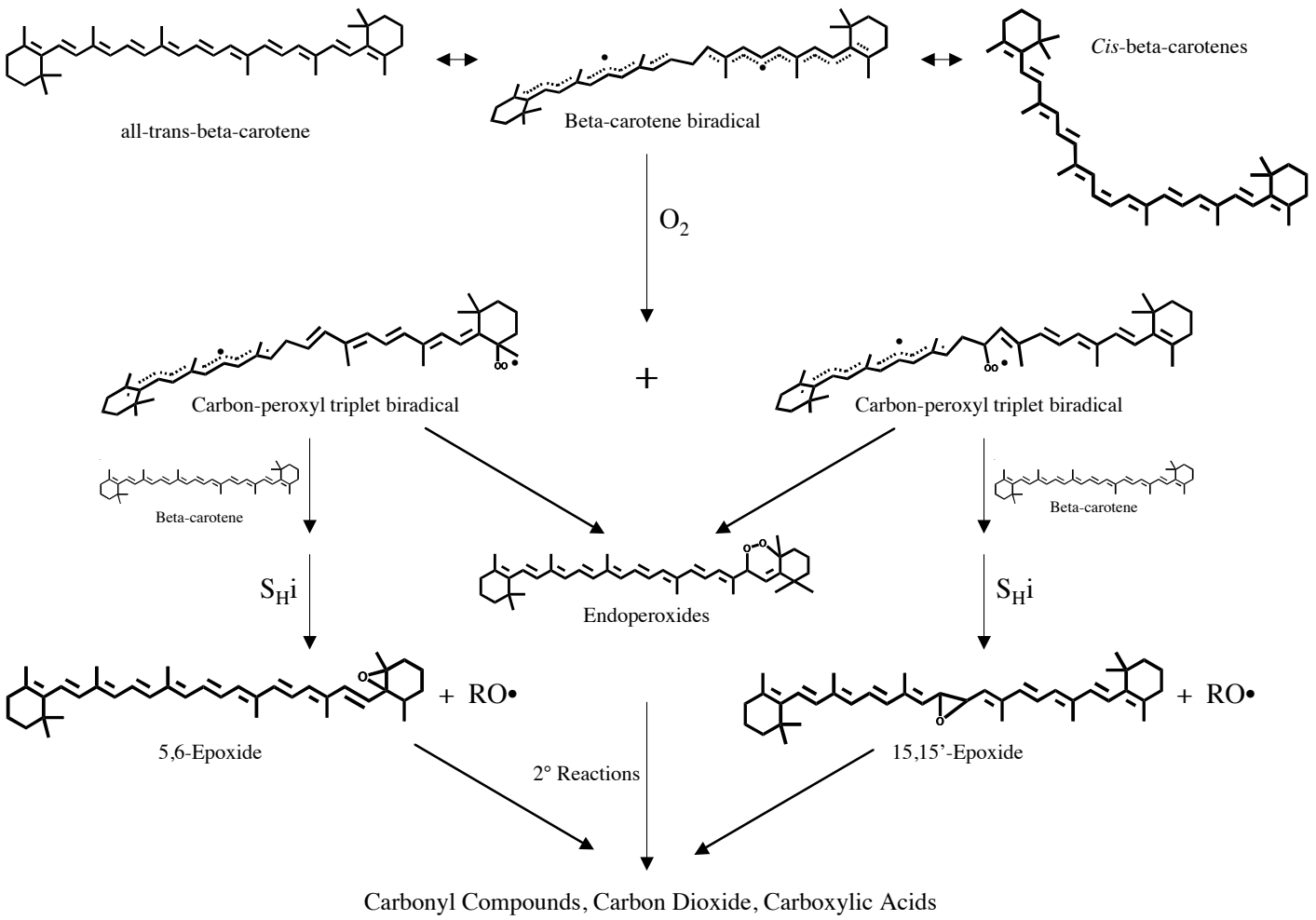

Figure 2.4: Possible pathways for the autoxidation of beta-carotene

CHAPTER 3

FACTORS IMPACTING LYCOPENE OXIDATION IN OIL-IN-WATER EMULSIONS

3.1 Introduction

Lycopene is a chromoplast carotenoid responsible for the red color of tomatoes, watermelon, guava, and grapefruit (12, 14). In recent years, a number of studies have suggested that dietary lycopene may decrease the risk for developing a number of health conditions due to its ability to act as an antioxidant and to stimulate cell-to-cell communication (32). The greatest evidence, thus far, for a medical condition that dietary lycopene impacts, is a reduced incidence of prostate cancer (139) . However, a number of studies have provided limited evidence that lycopene intake may also be associated with a reduced risk of cardiovascular disease (10) as well as cancers of the cervix, colon, esophagus, stomach, and breast (12, 13). These findings have increased consumer interest in lycopene-containing food products and generated new applications for lycopene-containing food ingredients.

The antioxidant properties associated with the health benefits of lycopene present challenges in preventing the degradation of lycopene within food products (140). Since lycopene is lipid-soluble (10), one way of protecting it from oxidative degradation might be to incorporate it into the oil phase of oil-in-water emulsions. Emulsions are effective ingredient delivery systems for functional foods because they can be engineered with numerous antioxidative functions (96). Antioxidative components can be incorporated

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into all three structural components of an emulsion (the aqueous phase, oil phase and the surfactant-containing interfacial region). can remain active once the emulsion is dispersed into water-based food (98, 105).

As a first step to understanding the potential for emulsions as oxidatively stable delivery systems for bioactive ingredients like lycopene, one must understand the relative oxidative susceptibility of each lipid within the emulsion. Food emulsions contain a variety of anti- and pro-oxidative species, which combined, influence the overall rate of degradation of both the fatty acids and bioactive components in emulsion droplets. In this work, various oil types were used to understand how oil composition might influence the rate of both fatty acid and lycopene oxidation. The results of this work should help to provide information on whether lipid oxidation or lycopene degradation is the most important factor in determining the shelf life of a lycopene-containing delivery system, as well as determining which oil composition might offer greatest protection.

When considering the oxidative stability of unsaturated fatty acids in oil-in-water emulsions, the reaction of most concern is the breakdown of lipid hydroperoxides naturally present at oil droplet interfaces by endogenous iron or other reactive transition metals in the aqueous phase (105). As this reaction proceeds, additional lipid hydroperoxides and highly reactive peroxyl and alkoxyl radicals are formed. These radicals in turn react with other unsaturated fatty acids thus propagating the oxidation reaction. Eventually, these reactions result in fatty acid decomposition to produce secondary oxidation products, such as aldehydes and ketones, which are associated with the off flavors and odors of rancid food products (62, 105, 123). By reducing the

interactions between lipid hydroperoxides and transition metals, the speed of the entire lipid oxidation process can be slowed (105).

A number of mechanisms have been used to reduce interactions between transition metals and lipid hydroperoxides in oil-in-water emulsions, including the manipulation of the interfacial region to form a physical and electrostatic barrier (104, 111, 131, 134, 141, 142). Mancuso et al. and Mei et al. (104, 111) showed that surfactant type could influence the rate of oxidation. Specifically, these studies found that emulsions stabilized by anionic surfactants oxidized at the fastest rates, followed by nonionic surfactants, while cationic surfactant-stabilized emulsions exhibited the slowest oxidation. The proposed reasoning for this observation was that cationic surfactants create a positively charged barrier around the oil droplets that repel cationic iron and other metals, while anionic surfactants attract iron to the droplet surface where it can easily degrade lipid hydroperoxides (104, 105, 111).

The objective of this work was to gain a better understanding of how the properties of the emulsion droplet interface will influence the oxidative stability of lycopene and unsaturated fatty acids. By gaining a better understanding of the mechanisms of lycopene degradation and lipid oxidation in emulsions, new antioxidant technologies could be developed that could stabilize this important bioactive component in functional foods.

3.2 Materials and methods

Corn oil was purchased from a local supermarket. LycoVit® Dispersion (11% lycopene) in sunflower oil was donated by BASF Corporation (Florham Park, NJ). Pure lycopene standard was a gift of LycoRed Ltd. (Beer Sheva, Israel).

Dodecyltrimethylammonium bromide (DTAB), sodium dodecyl sulfate (SDS), imidazole, barium chloride dihydrate, ammonium thiocyanate, iron(II) sulfate heptahydrate, silicic acid, activated charcoal, and *n*-hexane were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Polyoxyethylene(23) lauryl ether (Brij 35), sodium acetate, hydrochloric acid (HCl), sodium hydroxide (NaOH), iso-octane, 2-propanol, methanol, and 1-butanol were purchased from Fisher Scientific (Fair Lawn, NJ, USA). All chemicals were of analytical grade or purer.

3.2.1 Isolation of corn oil triacylglycerols

Stripped corn oil was prepared by diluting 30 g of oil with 30 ml of hexane. This mixture was passed through a chromatographic column (3.0 cm diameter, 35 cm in length). The bottom layer of the column was packed with 22.5 g of silicic acid that had been washed 3 times with distilled water, filtered, and activated at 110°C for 20 hours. A middle layer of 5.625 g of activated charcoal was used as well as a top layer of 22.5 g of the washed, filtered, and activated silicic acid. The oil was eluted with 270 ml of *n*-hexane and the solvent was removed in a Rotavapor RE 111 (Büchi, Postfach, Switzerland) at 38 °C. Traces of solvent were removed by flushing with nitrogen, and oil was stored at -80°C until use.

3.2.2 Preparation and storage of emulsions

Oil-in-water emulsions were prepared using 5% (w/w) oil phase in sodium acetate-imidazole buffer solution (10 mM each, pH 7.0) containing 30 mM surfactant (SDS, Brij 35, or DTAB). The oil phase of the emulsion was prepared by dispersing the

LycoVit® dispersion into corn oil, hexadecane, or stripped corn oil at a final concentration of 0.33 mg of lycopene per gram of oil (stored at -80°C until use). This amount of lycopene was chosen because it allowed for a reasonable range for quantifying lycopene using and integrating sphere. An aqueous phase was stirred overnight to ensure complete dispersion of the surfactant. Just prior to emulsion preparation, the oil phase was thawed using tap water. For corn oil emulsions, a coarse oil-in-water emulsion was prepared by blending the oil and aqueous phases for 2 minutes using a Biohomogenizer M133/1281-0 (Biospec Products, Inc., Bartlesville, OK, USA). Due to the increased physical instability of coarse emulsions containing hexadecane, these samples were prepared by blending for 2 minutes with the Biohomogenizer followed by an additional step of sonicating for 2 minutes (using 0.5 second pulses) at 70% amplitude using a Fisher Scientific Sonic Dismembrator 500. The coarse emulsions were then homogenized through a 2-stage high-pressure valve homogenizer (APV-Gaulin, Wilmington, MA, USA) for four passes at 4000 psi (27.6 MPa). During each pass, the emulsions were collected in a beaker submerged in a cool water bath. After homogenization, the pH of each emulsion was adjusted to pH 7.0 using HCl or NaOH. During each step in emulsion preparation, samples were covered as much as possible to reduce light exposure.

Emulsions were split into individual samples for each treatment and analysis, stored in the dark at 15°C, and rocked constantly. For experiments examining surfactant type, samples used to determine lycopene degradation were stored in 13 x 100 mm borosilicate glass test tubes. For experiments examining oil type, samples used to determine lycopene degradation were stored in 23-G-20 capped glass fluorometer cells (Starna Cells, Inc., Atascadero, CA, USA). Lipid hydroperoxide test samples were stored

in microcentrifuge tubes, and hexanal test samples were stored in 10 ml screw cap GC vials with PTFE/silicone septa.

3.2.3 Preparation and storage of pure lycopene emulsions

A series of lycopene degradation experiments were conducted to compare the degradation rates of pure lycopene to the LycoVit® dispersion, which contains sunflower oil in addition to the lycopene. To create the oil phase, pure lycopene crystals (95% purity) were added to hexadecane at a concentration of 3 mg lycopene/g hexadecane. The oil phase was then sonicated for 85 seconds (using 0.2 second on, 0.5 second off pulses) at 25% amplitude using a Fisher Scientific Sonic Dismembrator 500. The emulsions were then prepared as described above for hexadecane emulsions. Due to the expense of pure lycopene, smaller quantities of these emulsions were made, and samples were stored in 23-G-20 capped glass fluorometer cells (Starna Cells, Inc., Atascadero, CA, USA) and placed on rocker plates in the dark at 15ºC.

3.2.4 Physical properties of emulsions

Particle size distributions of the emulsion droplets were measured using a laser diffraction instrument (Malvern Mastersizer, Malvern Instruments, Worcestershire, UK). The mean particle diameters (D_{43}) of the DTAB, Brij 35, and SDS corn oil-in-water emulsions were found to be 0.36 ± 0.02 μm, 0.41 ± 0.11 μm, and 0.37 ± 0.01 μm, respectively. Hexadecane emulsions stabilized by SDS had a mean particle diameter (D_{43}) of 0.26 ± 0.01 μm. Particle size distributions were measured periodically and did not change over the course of the experiments. The electrical charge, or zeta potential

(ζ), of the emulsion droplets was measured using a micro-electrophoresis instrument (ZetaSizer Nano, Malvern Instruments, Worcestershire, UK). Zeta potential samples were prepared by diluting emulsions 1:100 with 10 mM sodium acetate-imidazole buffer (pH 7.0) and placing the dilutions into disposable capillary cells (Malvern Instruments, Worcestershire, UK).

3.2.5 Measurement of lipid oxidation

Lipid hydroperoxide concentrations were used as an indicator of primary lipid oxidation products. Lipid hydroperoxide concentrations were determined by a modified version of that described by Nuchi et al.(103). Emulsion samples (0.3 ml) were mixed with 1.5 ml of isooctane/2-propanol (3:1, v/v), by vortexing (10 s, 3 times). This mixture was then centrifuged at 3400 × g for 2 min. A 100 μL volume of the resulting organic solvent phase was added to 2.8 ml of methanol/1-butanol (2:1, v/v). Thiocyanate/ferrous solution (30 μL) (prepared by mixing equal volumes of 0.144 M $FeSO_4$ •7 H_2O with 0.132 M $BaCl_2$ (acidic solution), centrifuging, and mixing equal volumes of the clear ferrous solution with 3.94 M ammonium thiocyanate) was added to the methanol/1-butanol mixture, vortexed, and incubated at room temperature for 20 min. Following the incubation period, sample absorbances were read at 510 nm using a Genesys 20 spectrophotometer (ThermoSpectronic, Waltham, Massachusetts, USA). The hydroperoxide content was determined using a standard curve developed using known concentrations of cumene hydroperoxide.

Hexanal was measured as a secondary lipid oxidation product. Hexanal concentration was measured using a GC-17A Shimadzu gas chromatograph equipped

with an AOC-5000 autosampler (Shimadzu, Kyoto, Japan). A 30 m × 0.32 mm Equity DB-1 column (Supelco, Bellefonte, PA, USA) with a 1 μm film thickness was used for separations. Each sample was shaken and heated at 55°C in the autosampler heating block for 13 minutes. A 50/30 μm DVB/Carboxen/PDMS solid phase microextraction (SPME) fiber needle (Supelco, Bellefonte, PA, USA) was injected into the sample vial headspace for 1 minute to adsorb volatiles and then injected into the 250°C injector port for 3 minutes. The gas chromatograph ran for 10 minutes at 65°C for each sample. Helium was used as a carrier gas, with a total flow rate of 15.0 ml/minute. A flame ionization detector at a temperature of 250°C was used. Hexanal concentrations were determined from peak areas using a standard curve made from authentic hexanal.

Tocopherol content of oil phase samples were determined by normal-phase HPLC using refined vegetable oil preparation methods as described by Xu (143).

3.2.6 Lycopene concentration

The absorbance of lycopene containing emulsions was used to determine lycopene concentrations. Periodically, 7 ml sample volumes were placed in open glass fluorometer cells (20 x 10 mm) and measured using a Shimadzu UV-2101 PC UV-Vis scanning spectrophotometer equipped with an ISR – integrating sphere assembly (Shimadzu, Kyoto, Japan). For the pure lycopene degradation experiments, readings were taken using the same fluorometer cells in which the samples were stored. Lycopene content was determined using a standard curve created using various concentrations of corn oil emulsions with and without added lycopene. Concentrations were expressed

relative to zero time lycopene concentrations since small differences in lycopene concentrations occurred in the different emulsions.

3.2.7 Statistical analysis

All measurements were made using triplicate samples. Statistical Analysis Systems Version 9.1 software (SAS Institute, Cary, NC, USA, 2002) analysis of variance procedures (PROC GLM combined with the LS MEANS, SLICE and PDIFF functions) were used to analyze results. In this analysis, surfactant type, oil type, chelator addition, and storage time were considered fixed effects. Where significant interactions were found among the effects tested, a Bonferroni adjustment ($p \leq 0.05$/# of comparisons) was used for declaring significance. To determine when secondary oxidation products exited lag phase, a one-tailed Dunnett's Test was used to determine when hexanal concentrations were significantly higher than initial starting values.

3.3 Results and discussion

When examining the chemical stability of functional food delivery systems, it is essential to understand the stability of the bioactive ingredient as well as the overall oxidation profile of the lipid carrier. This is necessary to determine if the bioactive ingredient is degraded before lipid oxidation and thus the bioactive compound is lost prior to the product becoming rancid. Conversely, the bioactive lipid could oxidize slower than unsaturated fatty acids and thus the shelf-life would be determined by rancidity development instead of the stability of the bioactive lipid.

3.3.1 Impact of lycopene addition to corn oil emulsions

As a first step towards understanding the influence of lycopene on the oxidative profile of oil-in-water emulsions, two sets of emulsions were made. Both sets were stabilized by DTAB, Brij 35, or SDS. One set of emulsions was made with only corn oil as the lipid phase, while the other was made with corn oil containing 3 mg lycopene ingredient/g oil from a lycopene ingredient comprised of 11% lycopene in sunflower oil. The development of primary and secondary oxidation products in corn oil only and corn oil with added lycopene are shown in **Figures 3.1** and **3.2**, respectively. Formation of lipid hydroperoxides increased rapidly in oil-in-water emulsions stabilized with each of the emulsifiers in both emulsions without (**Figure 3.1A**) and with (**Figure 3.2A**) lycopene. Lipid hydroperoxide concentrations were lowest in Brij-stabilized emulsions, exhibiting significantly lower amounts than DTAB or SDS emulsions from 67-307 h in emulsions without lycopene and from 91-139 h in emulsions with lycopene. No consistent differences were found between emulsions stabilized by SDS and DTAB in lipid hydroperoxide formation. When the secondary lipid oxidation product, hexanal, was measured to monitor lipid oxidation in the emulsions without (**Figure 3.1B**) or with (**Figure 3.2B**) added lycopene, a lag phase was observed followed by a rapid rise in hexanal formation. Emulsions stabilized with SDS had much faster hexanal formation than the DTAB- and the Brij-stabilized emulsions both without (**Figure 3.1B**) and with lycopene (**Figure 3.2B**). In the SDS-stabilized emulsions without and with lycopene, the length of the lag phase of hexanal formation was only one testing point different (115 vs. 139 h, respectively). DTAB- and Brij-stabilized emulsions had longer lag phases of

hexanal formation, lasting 307 and 331 h, respectively in emulsions both with without lycopene (**Figures 3.1B** and **3.2B**).

The loss of lycopene was also measured in the emulsion with added lycopene (**Figure 3.3**). Lycopene degradation was fastest in SDS-stabilized emulsions and similar in DTAB- and Brij-stabilized emulsions. In all emulsions containing lycopene, the majority of lycopene was lost prior to the formation of hexanal. In the SDS-, DTAB- and Brij-stabilized emulsions approximately 90, 74 and 71% of the lycopene was degraded during the lag phase, respectively. Antioxidants that are effective at inhibiting lipid oxidation by scavenging free radicals do so by being preferentially oxidized thus delaying the oxidation of fatty acids and increasing the lag phase of the formation of lipid oxidation products. While lycopene was preferentially oxidized before fatty acids decomposed into hexanal, lycopene did not have a major impact on increasing the hexanal lag phase compared to emulsions without lycopene (**Figure 3.1** and **3.2**). This could occur if lycopene was interacting with free radicals, causing lycopene degradation, but resulting lycopene oxidation products (e.g. radicals) had sufficient energy to cause the oxidation of fatty acids thus not being able to inhibit lipid oxidation. Several other studies have shown that carotenoids can be antioxidative (135, 137) or proxidative in oil-in-water emulsions (135, 136, 144). Differences in the impact of carotenoids on oxidation rates in oil-in-water emulsions could be due to a number of factors including differences in storage conditions, concentrations and type of carotenoids and the presence of other antioxidants and proxidants.

Emulsions can be engineered to increase the oxidative stability of bioactive lipids within the emulsion droplet core. One such technique is to manipulate the surface charge

of the emulsion droplet and thereby alter the ability of transition metals to absorb to the emulsion droplet interface where they can efficiently promote oxidative reactions. **Table 3.1** shows the zeta potential of emulsions stabilized by DTAB, SDS and Brij 35. The oil-in-water emulsion droplets were found to be cationic when stabilized by DTAB and anionic when stabilized by SDS and Brij 35. SDS-stabilized emulsion droplets had a 14-fold higher negative charge density than emulsions stabilized by Brij 35. Zeta potential results were similar to those of previous studies using Brij and SDS as surfactants (145).

In these experiments, formation of lipid hydroperoxides did not vary greatly among the emulsions stabilized with the different emulsifiers. However, the SDS-stabilized emulsions had a much faster formation of hexanal. This pattern of anionic emulsion droplets having low lipid hydroperoxides and high secondary oxidation products such as hexanal has also been observed in other emulsions systems (109, 111, 112). This pattern is thought to be due to the high concentrations of transition metals associated with the anionic emulsion droplet interface, which leads to rapid hydroperoxide decomposition thus preventing hydroperoxide accumulation and causing rapid formation of hydroperoxide decomposition products such as hexanal.

The results from this study showed that cationic DTAB-stabilized emulsions droplets were not more oxidatively stable than emulsions stabilized with the slightly anionic Brij 35, a pattern not observed in other oil-in-water emulsion systems (104, 105, 111, 114, 131, 134). One possible reason for this discrepancy could be due to the higher levels of emulsifiers required to stabilize emulsions containing lycopene. When emulsions are made, surfactants absorb to the lipid surface until the surface becomes saturated. Remaining surfactant then partitions into the aqueous phase. If the

concentration of surfactant in the aqueous phase was above critical micelle concentration (CMC), the surfactant would form micelles. Brij 35 has lower CMC (0.09 mM (146)) than SDS or DTAB (6-8 mM (146) and 13.5 mM (147)). Since the emulsions in this work were prepared on an equal molar surfactant basis, it is more likely that Brij 35 would form more micelles in the continuous phase than SDS or DTAB. Since surfactant micelles have been shown to inhibit lipid oxidation (148, 149), this could explain why Brij 35- and DTAB-stabilized emulsions had similar oxidative stability.

3.3.2 Impact of oil type on stability of lycopene emulsions

To obtain a better understanding of how fatty acids and antioxidants in the oil phase influence lycopene oxidation in oil-in-water emulsions, lycopene containing SDS-stabilized emulsions were prepared with corn oil, corn oil stripped of its minor components, and hexadecane. Hexadecane, is a hydrocarbon that is fully saturated, and thus, is resistant to oxidation (148). **Figure 3.4A** shows the development of lipid hydroperoxides in the various treatments. The stripped corn oil samples oxidized most rapidly, forming significantly higher amounts of lipid hydroperoxides than all other samples within 20 hours of emulsion preparation. Corn oil samples with and without added lycopene exhibited similar lipid hydroperoxide formation with significant differences in lipid hydroperoxide concentrations only occurring at the final testing time (236 h). The hexadecane emulsion had no detectable lipid hydroperoxide throughout the experiment even though it contained a small amount of sunflower oil from the lycopene ingredient. Similar results were found when oxidation in the emulsions was analyzed by monitoring formation of the secondary oxidation product, hexanal (**Figure 3.4B**). Again,

the stripped corn oil with added lycopene oxidized first, with no substantial lag phase before the rapid rise in hexanal concentration. The corn oil only and corn oil with lycopene emulsions, exhibited similar lag phase behavior up to 68 h of storage, but during the exponential phase of hexanal formation, the corn oil only emulsions had a faster rate of hexanal formation than the corn oil with lycopene emulsions. This may suggest that while lycopene was not very effective in extending the lag phase, it may be able to quench alkoxy radicals to inhibit beta-scission reactions that would produce hexanal. Hexadecane samples, again, showed no lipid hydroperoxide or hexanal formation.

Lycopene degradation in emulsions with different oil types was in the order of stripped corn oil > hexadecane > corn oil (**Figure 3.5**). Lycopene degradation was so fast in the stripped corn oil that lycopene concentrations were significantly lower than the other two treatments within six hours of storage. The fact that lycopene degradation was faster in the stripped corn oil and hexadecane compared to corn oil suggests that antioxidants inherent in corn oil could be protecting the lycopene. Comparing lycopene degradation and hexanal development data shows that nearly 82 and 80% of the lycopene had been degraded before hexanal formation (**Figure 3.4B**) exited the lag phase in both stripped and non-stripped corn oil treatments, respectively.

The rapid lycopene oxidation observed in the hexadecane emulsions could be due to co-oxidation of the lycopene by oxidation of the sunflower oil in the lycopene ingredient. To test this, pure lycopene in hexadecane emulsions were made and lycopene degradation rates were compared when lycopene was added in the pure form or as lycopene in sunflower oil. The results of the degradation study in hexadecane can be

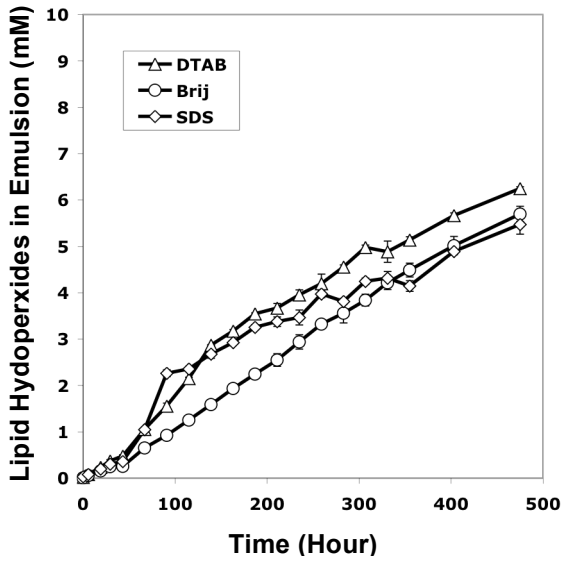
seen in **Figure 3.6**. These results clearly indicate that both forms of lycopene exhibited nearly identical degradation profiles. This suggests that the sunflower oil in the lycopene dispersion did not play a major role in the oxidation of lycopene in hexadecane-in-water emulsions containing the lycopene dispersed in sunflower oil (**Figure 3.5**). Instead, lycopene degradation in the hexadecane system might be caused by interactions with other emulsion components. Iron, which is commonly found in oil-in-water emulsions, has previously been shown to promote carotenoid degradation. In this pathway, ferric iron participates in an electron transfer reaction with carotenoids to form ferrous iron and a carotenoid radical cation, which is thought to further degrade by additional electron transfer reactions, deprotonation, or by reaction with oxygen (69-75, 87). Iron is known to be a strong proxidant in oxidation of unsaturated fatty acids in oil-in-water emulsions by the ability of ferrous ions to decompose lipid hydroperoxides into free radicals (105, 150). Therefore, in the corn oil based systems, iron may be recycling from ferric to ferrous during the reaction with lycopene, and then from ferrous to ferric upon reaction with lipid hydroperoxides. This recycling pathway could decrease the stability of both the fatty acids and the lycopene.

In corn oil-in-water emulsions containing lycopene it is likely that other oil components are protecting lycopene since lycopene degrades much faster in stripped corn oil that has had its minor components like tocopherols and carotenoids removed. This can be seen in **Figure 3.7** where total tocopherol concentrations in SDS-stabilized corn oil emulsions decreased prior to loss of lycopene and formation of hexanal. The rapid loss of tocopherols suggests that they are preferentially oxidized and thus could be protecting lycopene and unsaturated fatty acids from degradation. Tocopherols were

completely oxidized after 68 h of incubation at which time 46% of lycopene was lost and the hexanal concentrations were still in the lag phase (lag phase ended after 164 h). In addition to tocopherols other minor components in the oil such as phospholipids could also be inhibiting fatty acid and lycopene degradation (128)

Overall, this study suggests that oxidation of lycopene in oil-in-water emulsions is influenced by both surfactant and oil type. Interfacial characteristics of the emulsion droplet were also found to impact oxidation and lycopene degradation. Negatively charged interfaces on emulsions droplets (e.g. SDS) were found to produce the fastest oxidation rates, suggesting that cationic transition metals could be promoting lycopene degradation since they are attracted to the emulsion droplet surface where they can more readily interact with lycopene in the droplet interior. Lycopene was found to degrade more rapidly than unsaturated fatty acids but less rapidly than tocopherols. This suggests that in oil-in-water emulsions significant loss of lycopene could occur prior to the development of rancidity. Lycopene was also unstable in the absence of corn oil suggesting that the co-oxidation of lycopene by unsaturated fatty acids was unnecessary for lycopene degradation. In fact, components of corn oil actually inhibited the degradation of lycopene since lycopene degradation was faster in oils stripped of their minor components than unmodified oil. Since tocopherols are oxidized prior to lycopene, the preferential oxidation of tocopherols could be inhibiting the oxidation of lycopene. Overall, these results indicate that the stability of lycopene in oil-in-water emulsions could be altered by altering the physical properties of the emulsion droplet interface and by the addition of antioxidants such as tocopherols.

(A)



(B)

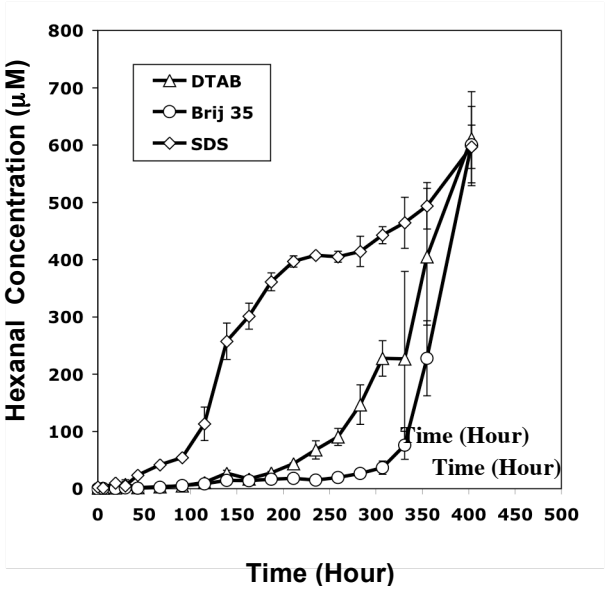
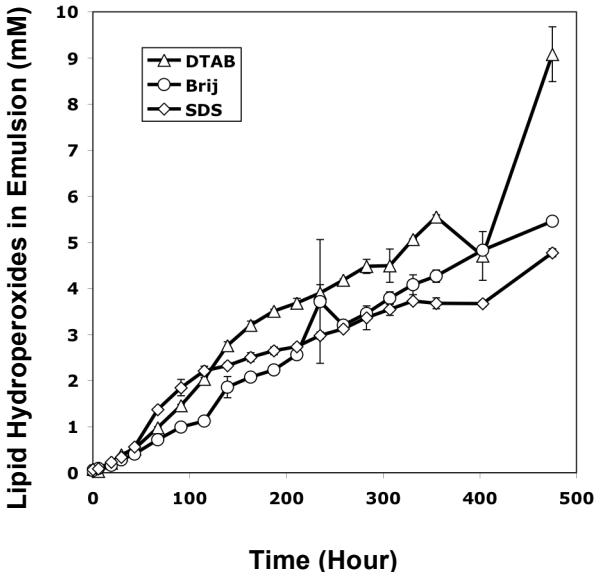


Figure 3.1: Development of lipid hydroperoxides (a) and hexanal (b) in corn oil oil-in-water emulsions stabilized by dodecyltrimethylammonium bromide (DTAB), polyoxyethylene(23) lauryl ether (Brij 35), or sodium dodecyl sulfate (SDS). Data points represent means (n=3) $\pm$ standard deviations. Some error bars lie within the data points.

(A)



(B)

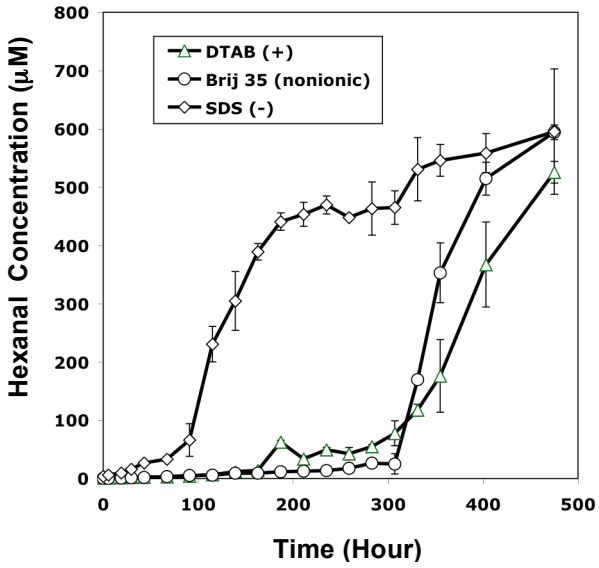


Figure 3.2: Development of lipid hydroperoxides (a) and hexanal (b) in corn oil oil-in-water emulsions containing lycopene stabilized by dodecyltrimethylammonium bromide (DTAB), polyoxyethylene(23) lauryl ether (Brij 35), or sodium dodecyl sulfate (SDS). Data points represent means (n=3) ± standard deviations. Some error bars lie within the data points.

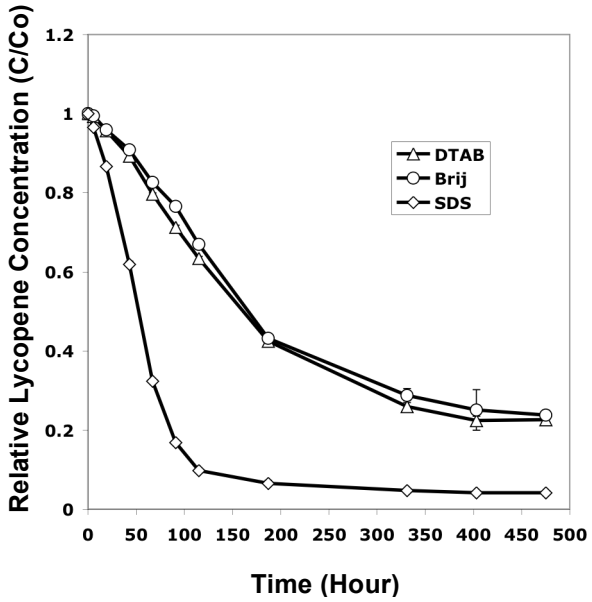
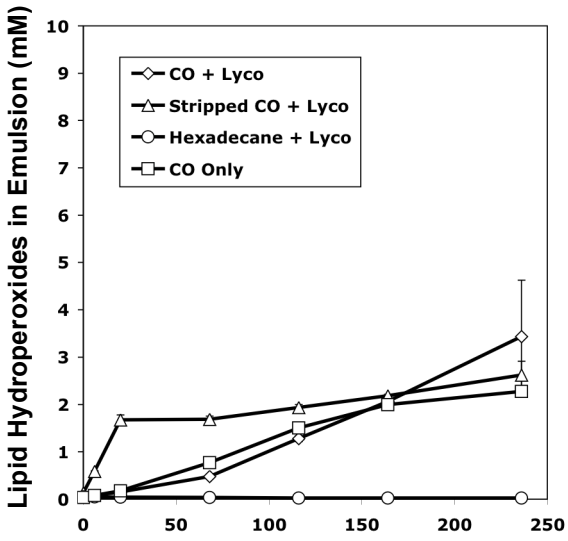


Figure 3.3: Relative lycopene concentration of dodecyltrimethylammonium bromide (DTAB), polyoxyethylene(23) lauryl ether (Brij 35), or sodium dodecyl sulfate (SDS)-stabilized corn oil oil-in-water emulsions. Data points represent means (n=3) $\pm$ standard deviations. Some error bars lie within the data points.

Table 3.1: Zeta potential of emulsions stabilized by small molecule surfactants.

SURFACTANT	ZETA POTENTIAL (mV)
DTAB	+46.1 ± 15.4
Brij 35	-3.2 ± 0.7
SDS	-107.6 ± 3.3

(A)



(B)

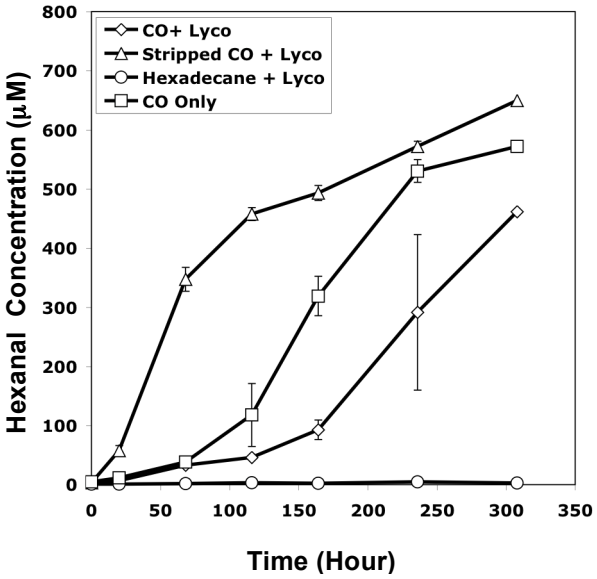


Figure 3.4: Development of lipid hydroperoxides (a) and hexanal (b) in sodium dodecyl sulfate-stabilized oil-in-water emulsions with oil phases comprised of either corn oil only, corn oil with added lycopene, stripped corn oil with lycopene, or hexadecane with lycopene. Data points represent means (n=3) ± standard deviations. Some error bars lie within the data points.

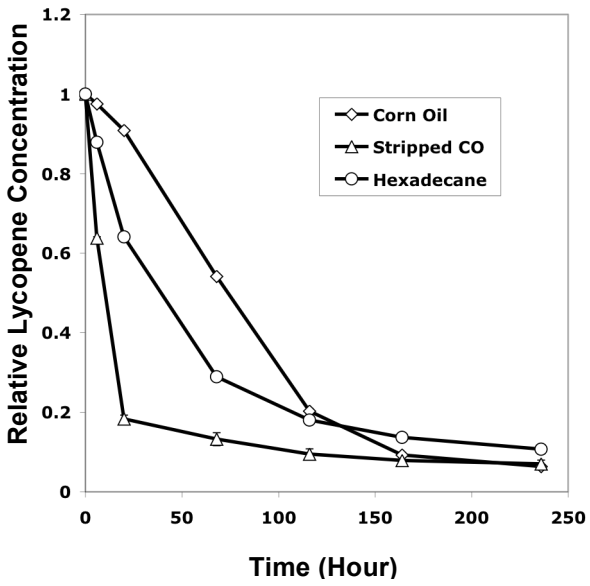


Figure 3.5: Relative lycopene concentration of sodium dodecyl sulfate-stabilized oil-in-water emulsions with oil phases comprised of corn oil with added lycopene, stripped corn oil with lycopene, or hexadecane with lycopene. Data points represent means (n=3) $\pm$ standard deviations. Some error bars lie within the data points.

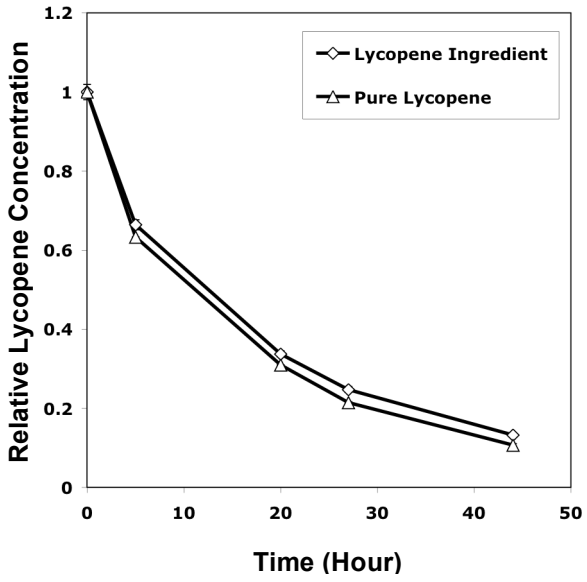


Figure 3.6: Relative lycopene concentration in sodium dodecyl sulfate-stabilized oil-in-water emulsions containing either pure lycopene or Lycovit® lycopene ingredient in hexadecane. Data points represent means (n=3) $\pm$ standard deviations. Some error bars lie within the data points.

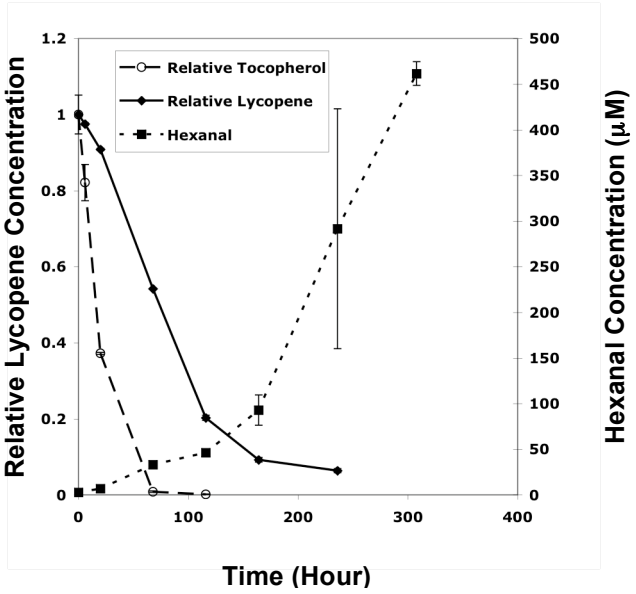


Figure 3.7: Relative tocopherol, relative lycopene, and hexanal concentrations in sodium dodecyl sulfate -stabilized corn oil-in-water emulsions with added lycopene. Data points represent means (n=3) $\pm$ standard deviations. Some error bars lie within the data points.

CHAPTER 4

MECHANISMS OF LYCOPENE DEGRADATION IN OIL-IN-WATER EMULSIONS

4.1 Introduction

In recent years research on the benefits of carotenoids in the diet has sparked interest in incorporating compounds such as lycopene and lutein into functional food products. While incorporating these bioactive compounds into foods that do not naturally contain high amounts of these compounds may offer great health benefits and provide new opportunities for food, there are a number of stability issues that must be overcome before these products can come to market.

The conjugated polyene chain that is characteristic of carotenoids makes these compounds susceptible to degradation from a number of agents. Autoxidation of carotenoids is known to occur with relative ease at room temperature in certain solvents, producing a number of initial oxidation products including carbon-peroxyl triplet biradicals and epoxides (48, 49). Exposure to high acid environments can cause destruction of carotenoids due to production of ion-pairs, which can then dissociate to form a carotenoid carbocation (67). Light (58, 59), heat (52-55, 57), and singlet oxygen (1, 60-62) are also known to degrade carotenoids.

Electron transfer has also been found to occur between transition metals like iron and carotenoids (68-76). If electron transfer occurs between a species like ferric iron and a carotenoid, the ferrous species of iron and a carotenoid radical cation can form (69, 70,

72-74). The resulting radical species formed, can undergo further degradation reactions, potentially leading to further carotenoid loss (73-75).

If free radicals are already present in the environment, three types of reactions have been found to occur between carotenoids and these radicals. Carotenoids have the potential to undergo electron transfer reactions with radicals in the environment to form radical cations (2, 73, 74, 80, 82, 83). In addition, carotenoids may react with radicals through hydrogen abstraction (62, 88, 89) or adduct formation reactions (29, 81, 88) that produce either carotenoid radicals or several types of radical adducts.

Once carotenoid degradation has been initiated by one of the mechanisms described above, a number of secondary reactions can occur leading to a variety of products including epoxides, endo-peroxides, apo-carotenals, and apo-carotenones (48, 49, 64, 74, 89, 94, 151). It is this series of reactions that can lead to both loss of color and bioactivity of carotenoids in foods, leading to loss of product quality and consumer acceptance.

This study focuses on the mechanisms of lycopene degradation in a oil-in-water emulsion model system. Lycopene is the acyclic carotenoid responsible for the red color of tomatoes, watermelon, guava, and grapefruit (12, 14). A number of studies have suggested that this compound may pay a role in decreasing the risk of developing a number of health conditions including cancers of the prostate (139), cervix, colon, esophagus, stomach, and breast (12, 13) as well as cardiovascular disease (10). It has been proposed that the benefits of lycopene are due to its ability to act as an antioxidant and to stimulate cell-to-cell communication (32).

Since carotenoids are lipid soluble, dispersing these compounds in the oil droplets of emulsions has great potential as a bioactive ingredient. Emulsion systems are easily incorporated into functional food products and can be designed to have several chemical protection hurdles that can increase stability of carotenoids. In previous work, emulsions were engineered to serve as a stable delivery system for omega-3 fatty acids in ice cream and yogurt, with little consumer detection of altered sensory attributes (96-99). The strategies that have been developed for increasing emulsion stability include engineering the oil/water interface, addition of antioxidants, and controlling the reactivity of prooxidant transition metals naturally present in foods through the use of metal chelators and product pH (97, 104, 108, 109, 111-113, 116, 119, 130, 131, 133).

In order to produce emulsion delivery systems for lycopene that exhibit optimum stability, it is critical to understand the predominant mechanisms of lycopene degradation. This work will examine the impact of factors known to play a role in carotenoid degradation (light, iron, free radicals, and pH), to determine the extent of damage these conditions create in a carotenoid-containing emulsion system. By identifying the mechanisms of lycopene loss, emulsions can be engineered to reduce interaction of carotenoids with prooxidative elements. Eventually, these strategies may make it possible to produce carotenoid delivery systems with adequate stability to be incorporated into functional food products.

4.2 Materials and methods

4.2.1 Materials

LycoVit® Dispersion (11% lycopene) in sunflower oil was donated by BASF Corporation (Florham Park, NJ). Sodium dodecyl sulfate (SDS), imidazole, sodium phosphate monobasic, tert-butylhydroquinone (TBHQ), 5,6-diphenyl-3-(2-pyridyl)-1,2,4-triazine-4',4''-disulfonic acid sodium salt (ferrozine), ferric chloride, ferrous sulfate, and hexadecane were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sodium acetate, hydrochloric acid (HCl), methanol, and sodium hydroxide (NaOH) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). All chemicals were of analytical grade or purer.

4.2.2 Preparation of oil phase

The oil phase of the emulsions was prepared immediately before use by dispersing the LycoVit® dispersion (11% lycopene in sunflower oil) into hexadecane at a final concentration of 0.33 mg of lycopene per gram of hexadecane. This amount of lycopene was chosen because it allows for a reasonable range for quantifying lycopene using an integrating sphere. In treatments using TBHQ, a methanol carrier was used to add TBHQ to the hexadecane. The methanol was evaporated with nitrogen before lycopene addition.

4.2.3 Preparation of emulsions

Oil-in-water emulsions were prepared using 5 % (w/w) oil phase in sodium acetate-imidazole-sodium phosphate buffer solution (10 mM each, pH 7.0) containing 30

mM SDS. An aqueous phase was prepared and stirred overnight to ensure complete dispersion of the surfactant. All emulsions were made at pH 7.0 by sonicating for 2 minutes, using 0.5 second pulses, at 70% amplitude using a Fisher Scientific Sonic Dismembrator 500. Emulsion preparation was conducted in the dark to prevent any potential degradation by light. All glassware used for emulsion preparation and sample storage was acid washed with 2.0 N HCl to remove residual metals. After sonication, the pH was adjusted to the appropriate treatment pH. For EDTA treatments, EDTA was added to the aqueous phase prior to emulsification at a final concentration of 100 mM.

4.2.4 Sample storage

Samples were stored in 23-G-20 capped glass fluorometer cells (Starna Cells, Inc., Atascadero, CA, USA) placed on rocker plates, and stored at 15ºC or room temperature (22-25ºC). With the exception of experiments testing the effect of light, all samples were stored in the dark.

4.2.5 Influence of light

To understand the ability of light to degrade lycopene, samples at various pH levels stored in light or dark were tested for lycopene degradation over time. Samples in the light treatment were stored on a rocker plate under a 60 Hz fluorescent lamp fixture. For comparison, a second set of samples were stored on rocker plates and covered to prevent light exposure. All samples were stored at 15ºC. All emulsion preparation procedures and lycopene concentration measurements were made in the dark.

4.2.6 Influence of ferric and ferrous species of iron

To determine the effect of iron species on the stability of lycopene in emulsions, ferric chloride or ferrous sulfate stock solutions were added to emulsion samples at a final concentration of 100 uM. For samples containing no iron, distilled, deionized water was added to the emulsions. The effects of ferrous and ferric iron were also tested in bulk hexadecane with lycopene. For this work, a methanol carrier was used to add ferric chloride or ferrous sulfate to the hexadecane at a final concentration of 100 uM ferrous or ferric species. Lycopene was added to give a final concentration of 11 uM in the hexadecane. After stirring for five minutes to incorporate the lycopene into the hexadecane, lycopene concentration was determined.

4.2.7 Particle size of emulsions

Particle size distributions of the emulsion droplets were measured using a laser diffraction instrument (Malvern Mastersizer, Malvern Instruments, Worcestershire, UK). Particle size was determined on triplicate samples for each emulsion preparation. The average D_{43} of the emulsions was 0.4 ± 0.02 μm and the average D_{32} was 0.28 ± 0.02 μm. Particle sizes were stable throughout the storage studies.

4.2.8 Lycopene concentration

The absorbance of emulsions was used to determine lycopene concentrations. Periodically, sample absorbances were measured using a Shimadzu UV-2101 PC UV-Vis Scanning Spectrophotometer equipped with an ISR – Integrating Sphere Assembly (Shimadzu, Kyoto, Japan). Lycopene content was determined using a standard curve

created using emulsions with varying amounts of added lycopene. Duplicate samples were tested for each treatment at each testing time.

To determine lycopene concentration for the bulk oil experiment in which ferric and ferrous iron were added, the absorbance of the hexadecane and lycopene mixture was read at 470 nm using a Shimadzu UV-2101 PC UV-Vis Scanning Spectrophotometer. Triplicate samples were tested at each testing time. Lycopene content was determined from a standard curve prepared with lycopene and hexadecane.

4.2.9 Ferrous and ferric iron content

Ferrous iron content was determined using ferrozine chelation, a method initially developed by Stookey (152). A ferrozine solution (3 mM final concentration) was added directly to emulsion samples and absorbance was measured at 562 nm using a Shimadzu UV-2101 PC UV-Vis Scanning Spectrophotometer equipped with an ISR – Integrating Sphere Assembly (Shimadzu, Kyoto, Japan). Emulsion ferrous iron concentration was determined using standard curves prepared from ferrous sulfate.

4.2.10 Statistical analysis

Statistical Analysis Systems Version 9.1 software (SAS Institute, Cary, NC, USA, 2002) for analysis of variance procedures (PROC GLM combined with the LS MEANS, SLICE and PDIFF functions) was used to analyze results. In this analysis, pH, light, iron type, chelator addition, free radical scavenger addition, and storage time were considered fixed effects. Where significant interactions were found among the effects tested, a Bonferroni adjustment ($p \leq 0.05/\#$ of comparisons) was used for declaring significance.

4.3 Results and Discussion

The objective of this work was to gain a better understanding of the major mechanisms of lycopene degradation in oil-in-water emulsions to help to identify which food processing, formulation, and storage conditions might be important to consider for a lycopene-containing emulsion based functional food ingredient. Factors evaluated included light exposure, free radical scavengers and iron since these factors have been found in previous studies to decrease the stability of carotenoids *in vitro*.

4.3.1. Impact of light

The influence of light on the stability of lycopene in emulsions was determined by splitting emulsion samples into two groups. One group was stored in the dark at 15°C, and the other was stored under fluorescent lighting at the same temperature. Fluorescent lighting was chosen as it is common in many food markets. Emulsion samples at pH 3.0, 5.0, and 7.0 that were stored in either the dark or light showed little difference in lycopene loss over storage (**Figure 4.1**). At pH 3, both light and dark storage conditions resulted in a near total (>99%) loss of lycopene after 70 hours of storage. Significant differences between samples stored in light or dark conditions were found only at the 28 and 46 hours sampling intervals. Samples at pH 5.0 and 7.0, showed a greater stability than emulsion samples at pH 3.0. Over the course of the experiment (94 hours), samples stored at pH 5.0 or 7.0, experienced a 45-50% lycopene loss. No significant differences were found between the samples stored in the dark or under fluorescent lighting at pH 5.0. Significant differences were found at several testing times at pH 7.0 however, these differences were not consistently higher or lower suggesting

that there is little difference in lycopene stability in emulsions stored in the light and dark at pH 7.0.

Lack of significant lycopene degradation in the presence of light could be due to several reasons. First, the lipid droplets in oil-in-water emulsions will scatter light (101), which may result in limited penetration of the light into the sample. Unlike other studies which studied light degradation of carotenoids in optically clear solvents (59), the lack of light penetration would mean that most of the lycopene in the oil-in-water emulsion was not exposed to light thus decreasing its degradation. Second, light can be very efficient in degrading carotenoids in the presence of singlet oxygen generators (1, 60-62). However, since the oil-in-water emulsions used in this study did not contain singlet oxygen generators such as riboflavin or chlorophyll, this pathway is unlikely to be an important mechanism of lycopene degradation. This may not be the case if a lycopene-containing oil-in-water emulsion was added to foods containing singlet oxygen generators.

4.3.2 Impact of free radical scavengers

Carotenoids can also degrade in the presence of free radicals (2, 29, 62, 73, 74, 80-83, 88, 88, 89). Therefore, TBHQ, a free radical scavenger known to be effective in oil-in-water emulsions (153, 154) was added to the emulsions using stock solutions made with methanol. Final concentrations of TBHQ emulsions were 200 or 500 ppm, while control samples were made by adding an equivalent volume of methanol without any TBHQ. TBHQ addition was found to have little impact on lycopene stability in emulsions at pH 3.0 stored at 15°C (**Figure 4.2**) as all samples exhibited near complete

loss of lycopene within 140 hours of storage. At pH 7.0 TBHQ was more effective in reducing lycopene loss than at pH 3.0. Within 52 hours of storage, pH 7.0 samples without added TBHQ exhibited significantly greater lycopene loss than samples with either 200 or 500 ppm TBHQ. At the conclusion of the experiment, the samples without TBHQ exhibited a 44% lycopene loss, while those with TBHQ exhibited only a 14-17% loss.

Carotenoids can degrade through free radical mediated autooxidation reactions (2, 80, 81). The lycopene used in this study was suspended in sunflower oil (0.27% of the total lipids in the emulsion). While the concentration of sunflower oil in the emulsion is low, it is possible that its degradation could produce free radicals that could in tum degrade lycopene. The fact that TBHQ was effective at pH 7.0 but not 3.0 suggests that free radicals were the predominant mechanism for lycopene degradation at pH 7.0. The ineffectiveness of TBHQ at pH 3.0 could be due to lack of significant free radical production at lower pH values or could be due to the high reactively of other prooxidants that would rapidly degrade TBHQ making it ineffective.

4.3.3 Impact of pH

The previous experiments indicated that the pH of the emulsion appears to have a dramatic impact on lycopene stability. To further examine how the stability of lycopene in oil-in-water emulsions is influenced by pH, emulsions were prepared at pH 2.0-8.0 and stored at 15°C (**Figure 4.3**). Emulsions at pH 2.0-4.0 exhibited near total loss of lycopene (96-99% loss) over the course of the 94 hour experiment. Alternatively, the pH

5.0-8.0 emulsions exhibited a much slower rate of lycopene loss, resulting in a total lycopene loss between 47-54% after 94 h of incubation.

In strong acids like trifluoroacetic and sulfuric acid, carotenoids have been proposed to produce ion pairs, leading to destruction of carotenoids (67, 68). However, acid-catalyzed reactions may not be the predominant mechanism by which lycopene stability is decreased with decreasing pH. Also at play may be the solubility of iron. Iron has been shown to degrade several carotenoids in various solvent systems (68-76). Since ferric iron increases in solubility from 1.6×10^{-18} M to 1.6 M as pH decreases from pH 8.0 to pH 2.0 and ferrous iron increases in solubility from 3.7×10^{-3} to 3.7×10^{9} M over the same range (155), it would be expected that iron-induced reactions would become more prominent at lower pHs. Increased prooxidant activity of iron with decreases in pH has previously been observed in the oxidation of fatty acids and fatty acid hydroperoxides in oil-in-water emulsions. For example, lipid hydroperoxides in SDS-stabilized emulsions and in Tween 20 micelles were proposed to experience a more rapid breakdown by iron at pH 3.0 than at pH 7.0 due to greater iron solubility at low pH (102, 102, 111). Low pH could not only impact iron solubility but could also impact the physical location of iron in emulsions. Mei and coworkers (1998) reported that the association of ferric iron with SDS-stabilized hexadecane-in-water emulsions increased with decreasing pH. Since binding of iron to the surface of emulsion droplets can increase oxidation rates by increasing metal-lipid interactions (112), this could also help explain why lycopene degradation increased with decreasing pH.

4.3.4 Impact of EDTA and iron

To determine if metals were active at promoting lycopene degradation in the oil-in-water emulsions, 100 uM EDTA was added (**Figure 4.4**). Samples containing EDTA, exhibited significantly greater stability than those without EDTA after 3 hours for samples at pH 3.0 and 22 hours for samples at pH 5.0 and 7.0. EDTA was more effective at protecting lycopene at pH 3.0 and 5.0 than pH 7.0, however, no significant differences in lycopene stability were observed in the presence of EDTA between pH 3.0 and 5.0 samples. This data provides strong evidence that transition metals such as iron and copper are highly involved in lycopene degradation in the oil-in-water emulsion especially at lower pH values.

Of the transition metals in foods, iron is an important prooxidant because it is typically found at greater concentrations than the other transition metals. To provide greater clarity to the question of the role of iron and specific iron species on lycopene degradation, ferrous or ferric ions were added to the emulsions. Ferrous sulfate (100 uM) increased the stability of lycopene in emulsions both at pH 3.0 and 7.0 (**Figure 4.5**), with ferrous-containing samples becoming significantly more stable than the other treatments within three hours at pH 3.0, and within 24 hours at pH 7.0. The reason for the higher stability of lycopene in ferrous treated oil-in-water emulsions is not clear. Ferrous iron has been shown to act as an antioxidant at high concentrations in some systems. For instance, ferrous acetate (50 ppm) addition to an extrudate of corn starch and soybean oil was found to reduce lipid oxidation compared to a no iron added control (156). In non-food systems, some metal compounds have also been shown to act both as an inhibitor and promoter of oxidation reactions depending on their concentration. Betts and Uri first

proposed that various cobalt compounds act to catalyze oxidation of peroxide-free methyl linoleate, docos-1-ene, hexadec-1-ene, and 2,6,10,14-tetramethylpentadecane at low concentrations, but convert to inhibitors of oxidation at higher concentrations (157). It has been proposed that this antioxidant phenomenon occurs at high levels of transition metals because electrons from reduced metal ions can inactivate free radicals.

At pH 3.0, addition of ferric chloride (100 uM) resulted in a rapid loss of lycopene under room temperature storage (23-25°C) (**Figure 4.5**). Within the first hour of storage, 83% of lycopene present in the emulsion was lost. Emulsions at pH 3.0 without added iron, experienced the second fastest loss of lycopene, with a 69% decrease in lycopene concentrations after 24 h of storage. Ferric chloride addition did not accelerate lycopene degradation at pH 7.0. The rapid degradation of carotenoids upon exposure to ferric ions has been reported for beta-carotene, canthaxanthin, and several apo-carotenoids in studies using carotenoids and ferric chloride in dichloromethane (69, 71-76) or sol-gels containing ferric ions in the aqueous fraction (68). The increased activity of ferric iron at pH 3.0 is likely due to higher solubility making interactions between aqueous phase iron and oil droplets more likely.

The ability of ferric and ferrous iron to degrade lycopene was also tested in bulk hexadecane containing lycopene (**Figure 4.6**). As in emulsion samples, ferric iron caused a rapid destruction of lycopene, with only 10% of lycopene remaining after 165 hours of storage. The lycopene in treatments with no iron added or ferrous iron added, exhibited greater stability, and no significant differences were found between these two treatments.

Ferric ions have been proposed to degrade carotenoids by the following mechanism:

$$Fe^{3+} + \text{carotenoid} \rightarrow Fe^{2+} + \text{carotenoid}^{\bullet+} \quad (4.1)$$

To determine if ferric was converted to ferrous ions in the emulsion system used in this study, a ferrous ion indicator, ferrozine, was added to the samples. Ferric chloride (or distilled, deionized water, in the case of the no iron control) was then added to the emulsions at a final concentration of 100 uM and formation of ferrous ions was monitored at room temperature for 1000 s at 562 nm. As seen in **Figure 4.7**, oil-in-water emulsions containing lycopene caused a rapid conversion of ferric to ferrous ions with over 90% of the ferric iron being lost within 300 seconds. When lycopene was not present in the emulsion, only minor amounts ferric ions were converted to ferrous ions during the 1000 s incubation period. These results strongly suggest that the mechanism described in Equation 1, is responsible for the initiation of lycopene degradation at low pH levels.

4.4 Conclusion

It is clear from the work conducted in these experiments that lycopene in SDS-stabilized emulsions can undergo two degradation pathways, and it is possible that these two pathways can occur simultaneously. At low pH, where iron is more soluble, the predominant pathway of lycopene destruction is likely due to interactions with ferric ions. However, as the pH of emulsions is raised, iron may be less likely to interact with the lipid droplets due to loss of solubility and therefore ability to interact with emulsion droplets. When iron-induced degradation pathways are less frequent, free radical induced

lycopene degradation becomes more dominant. In the hexadecane emulsion system, the free radicals are likely generated by autooxidation, from the minor amounts of sunflower oil present in the BASF lycopene ingredient, or from carotenoid radical cations produced by a limited number of iron-lycopene interactions that still take place at high pH.

This situation could change if a lycopene-containing emulsion was added to a real food system. For example, in lipid containing foods, iron is likely to react with both lycopene and naturally occurring lipid hydroperoxides. The reaction between iron and lipid hydroperoxides would result in generation of free radicals and ferric ions. Both the ferric iron and the free radicals could then attack lycopene, making both free radical and ferric ion–promoted degradation a concern especially in low pH foods. If the pH of a food product is higher, iron will be less soluble, in which case, free radical reactions may become the more important pathway of degradation.

Therefore, when designing functional foods containing lycopene emulsions it would be important to add metal chelators and/or free radical scavengers. In high pH foods, free radical scavengers may be sufficient to inhibit lycopene degradation. In low pH foods, metal chelators would help stabilize the lycopene. In foods containing unsaturated fatty acids, it might be advisable to add both chelators and free radical scavengers to inhibit interactions between iron and fatty acid hydroperoxides and inactivate any free radicals produced by hydroperoxide decomposition. This work also provides strong evidence that ferric ion concentrations should be keep as low as possible in lycopene-containing foods and supplements especially those with acidic pH values. It may also be possible to design lycopene delivery systems that have emulsion droplet

interfaces engineered to inhibit metal-lycopene interactions (e.g. emulsion droplets with thick or cationic interfacial membranes that repel iron).

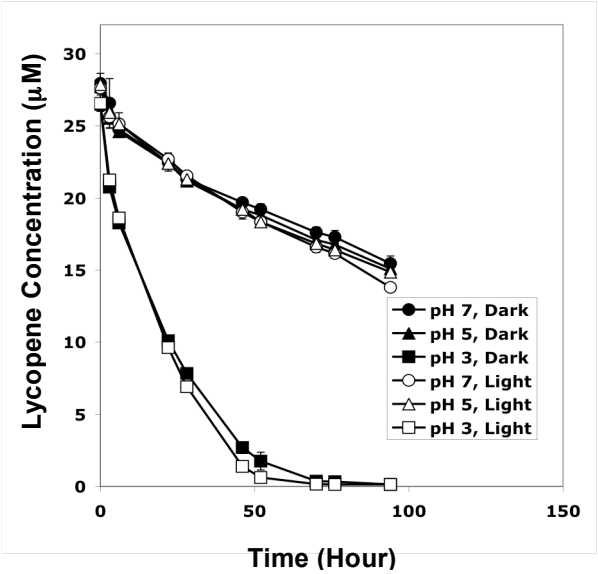


Figure 4.1: Lycopene concentration over storage time in SDS-stabilized emulsions at pH 3.0, 5.0, or 7.0 stored in light or dark conditions at 15°C. Data points represent means (n=3) $\pm$ standard deviations. Some error bars lie within the data points.

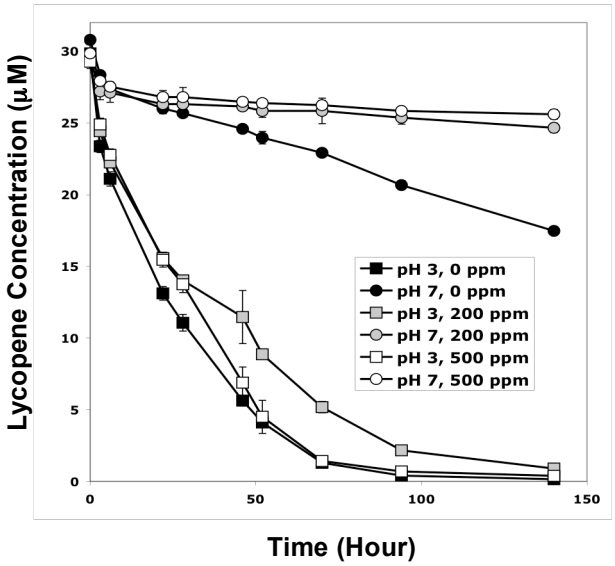


Figure 4.2: Lycopene concentration over storage time in SDS-stabilized emulsions at pH 3.0 or 7.0 stored 15°C with 0, 200, or 500 ppm TBHQ. Data points represent means (n=3) $\pm$ standard deviations. Some error bars lie within the data points.

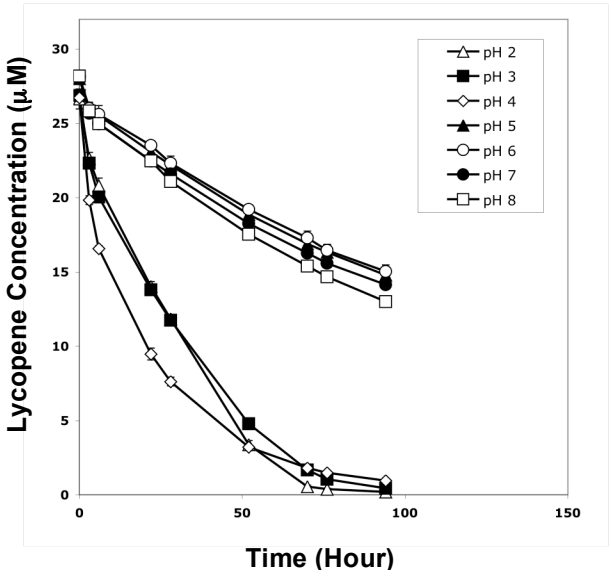


Figure 4.3: Lycopene concentration over storage time in SDS-stabilized emulsions at pH 2-8 stored at 15°C. Data points represent means (n=3) ± standard deviations. Some error bars lie within the data points.

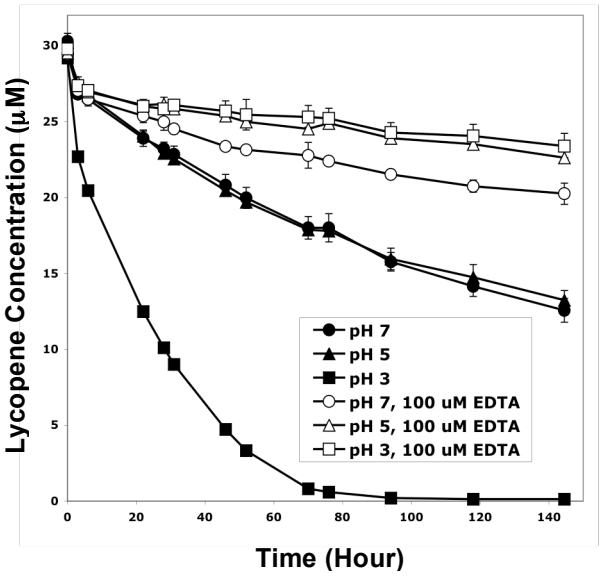


Figure 4.4: Lycopene concentration over storage time in SDS-stabilized emulsions at pH 3.0, 5.0, or 7.0 stored at 15°C with 0 or 100 uM EDTA. Data points represent means (n=3) $\pm$ standard deviations. Some error bars lie within the data points.

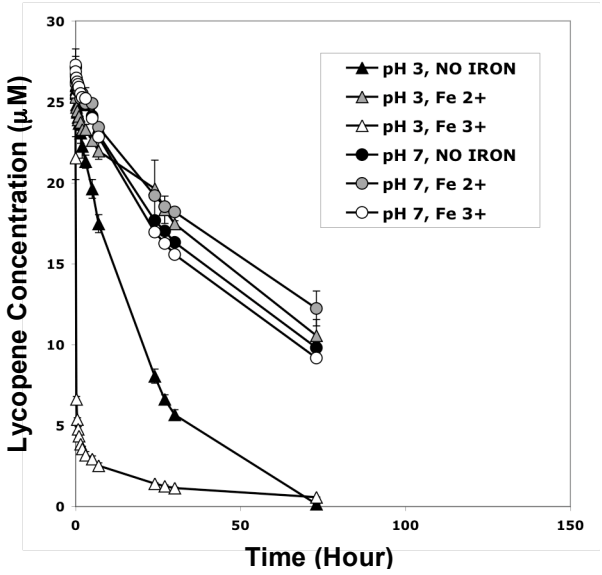


Figure 4.5: Lycopene concentration over storage time at room temperature in SDS-stabilized emulsions at pH 3.0 or 7.0, with 100 uM ferrous iron, 100 uM ferric iron, or no iron added. Data points represent means (n=3) $\pm$ standard deviations. Some error bars lie within the data points.

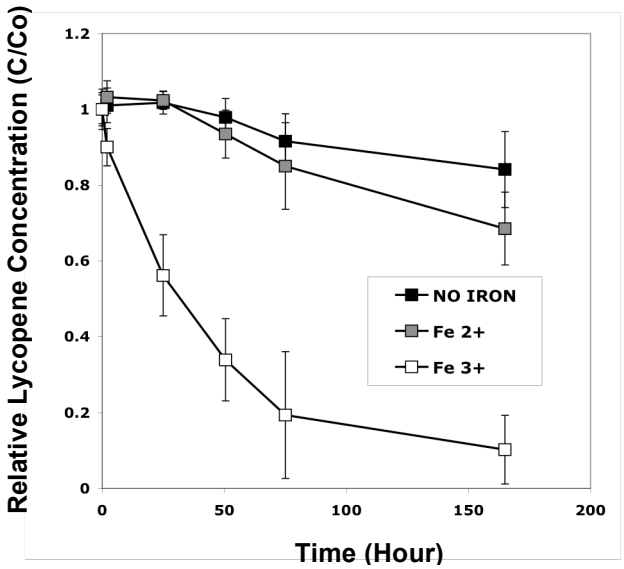


Figure 4.6: Lycopene concentration over storage time at room temperature in hexadecane with 11 uM lycopene and 100 uM ferrous iron, 100 uM ferric iron, or no iron added. Data points represent means (n=3) $\pm$ standard deviations. Some error bars lie within the data points.

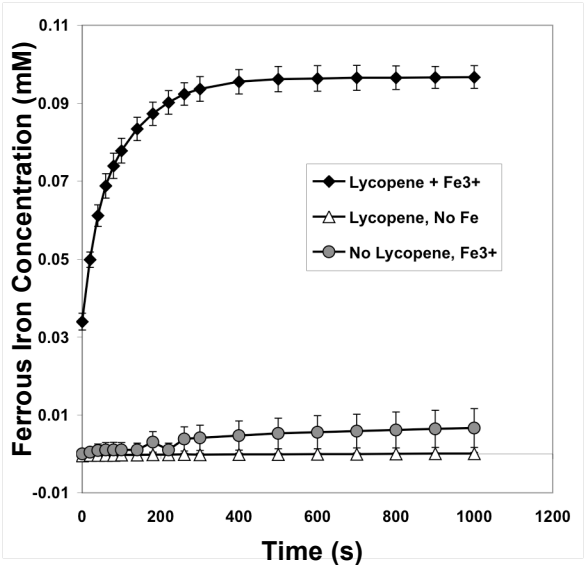


Figure 4.7: Ferrous iron development over time in SDS-stabilized emulsions with lycopene and 100 uM ferric iron added, with lycopene and no iron added, or with no lycopene and 100 uM ferric iron added. Data points represent means (n=3) + standard deviations. Some error bars lie within the data points.

CHAPTER 5

ROLE OF IRON AND HYDROPEROXIDES IN THE DEGRADATION OF LYCOPENE IN OIL-IN-WATER EMULSIONS

5.1 Introduction

Research on the health benefits of carotenoids in the diet has sparked interest in incorporating these compounds into functional food products. While incorporating these bioactive compounds into foods that do not naturally contain high amounts of these compounds may improve health and provide new markets for food products, there are also a number of stability issues that must be overcome. The conjugated polyene chain that is characteristic of carotenoids makes these compounds susceptible to degradation from a number of agents including transition metals (68-76), free radicals (2, 29, 62, 73, 74, 80-83, 88, 89), high acid environments (67), light (58, 59), heat (52-55, 57), singlet oxygen (1, 60-62), and autoxidation (48, 49).

Previous research in our laboratory has shown that transition metals and to a lesser extent free radicals, play a dominant role in the degradation of lycopene in oil-in-water emulsions stabilized by sodium dodecyl sulfate (SDS). The ferric species of iron was found to promote rapid destruction of lycopene in SDS-stabilized emulsions and in bulk hexadecane containing added lycopene (158). This lycopene degradation was most likely caused by the reaction of the neutral lycopene with the ferric species to produce a lycopene radical cation and the ferrous species of iron (Equation 5.1), which has been shown to occur in several studies (69, 70, 72-74).

$$Fe^{3+} + \text{lycopene} \rightarrow Fe^{2+} + \text{Lycopene}^{\bullet+} \quad (5.1)$$

Radical species may also react with neutral lycopene by several different mechanisms including electron transfer, hydrogen abstraction, and adduct formation, to form a number of degradation products, some of which are themselves reactive, leading to a cycle of degradation reactions (2, 29, 62, 73, 74, 80-83, 88, 89).

The intent of this study is to expand understanding of the mechanisms of lycopene degradation in a model oil-in-water emulsion system. Lycopene is an acyclic carotenoid responsible for the red color of tomatoes, watermelon, guava, and grapefruit (12, 14). A number of studies have suggested that this compound may pay a role in decreasing the risk of developing a number of health conditions including cancers of the prostate (139), cervix, colon, esophagus, stomach, and breast (12, 13) as well as cardiovascular disease (10). It has been proposed that the benefits of lycopene are due to its ability to act as an antioxidant and to stimulate cell-to-cell communication (32).

Since carotenoids are lipid soluble, dispersing these compounds in the oil droplets of emulsions has great potential as a food ingredient. Emulsion systems allow for both easy incorporation into functional food products as well as opportunities to incorporate various strategies to increase stability of carotenoids. In previous work by our group, emulsions have been engineered to serve as a stable delivery system for omega-3 fatty acids in ice cream and yogurt, with little consumer detection of altered sensory attributes (96-99).

The objective of this work is to examine the role of ferric and ferrous iron on the stability of lycopene in oil-in-water emulsions. This will expand upon the initial work in this area in which lycopene dispersed in hexadecane was incorporated into an emulsion stabilized by the anionic surfactant SDS. In this work however, the emulsions will be

stabilized by the nonionic surfactants, Brij 35 and Tween 20, to examine whether the same degradation patterns will occur in oil-in-water emulsions where the emulsion droplets do not have a high negative charge.

5.2 Materials and methods

5.2.1 Materials

LycoVit® Dispersion (11% lycopene) in sunflower oil was donated by BASF Corporation (Florham Park, NJ). Sodium dodecyl sulfate (SDS), imidazole, sodium phosphate monobasic, ferrous sulfate heptahydrate, barium chloride dihydrate, polyoxyethylene (20) sorbitan monolaurate (Tween 20), ammonium thiocyanate and hexadecane were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Polyoxyethylene (23) lauryl ether (Brij 35), sodium acetate, hydrochloric acid (HCl), methanol, ferric chloride hexahydrate, 1-butanol, and sodium hydroxide (NaOH) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). All chemicals were of analytical grade or purer.

5.2.2 Preparation of emulsions

Oil-in-water emulsions were prepared using 5 wt% oil in sodium acetate-imidazole-sodium phosphate buffer solution (10 mM each, pH 7.0) containing 30 mM SDS, Brij 35, or Tween 20 . The aqueous phase was prepared and stirred overnight to ensure complete dispersion of the surfactant. The oil phase of the emulsion was prepared immediately before use by dispersing the LycoVit® dispersion into hexadecane at a final concentration of 0.33 mg of lycopene per gram of hexadecane. This amount of lycopene

was chosen because it allowed for a reasonable range for quantifying lycopene using an integrating sphere. All emulsions were made at pH 7.0 by sonicating for 2 minutes (using 0.5 second pulses) at 70% amplitude using a Fisher Scientific Sonic Dismembrator 500. All glassware used for emulsion preparation and sample storage was acid washed with 2.0 N HCl to remove residual iron. After sonication, pH was adjusted to 3.0.

5.2.3 Emulsion sample storage

Samples were stored in 23-G-20 capped glass fluorometer cells (Starna Cells, Inc., Atascadero, CA, USA) and placed on rocker plates. All samples were stored at room temperature (25-27ºC) in the dark.

5.2.4 Physical properties of emulsions

Particle size distributions of the emulsion droplets were measured using a laser diffraction instrument (Malvern Mastersizer, Malvern Instruments, Worcestershire, UK). Particle size was determined on triplicate samples for each treatment after emulsion preparation and at the end of each experimental run. Mean particle size of emulsions (D_{32}) stabilized by SDS, Brij 35, or Tween 20 were 0.28 ± 0.02 μm, 0.23 ± 0.01 μm, and 0.22 ± 0.01 μm respectively. No differences in particle size were found over the course of the study.

The electrical charge, or zeta potential (ζ), of the emulsion droplets was measured using a micro-electrophoresis instrument (ZetaSizer Nano, Malvern Instruments, Worcestershire, UK). Zeta potential samples were prepared by diluting emulsions 1:100

with 10 mM sodium acetate-imidazole buffer (pH 7.0) and placing the dilutions into disposable capillary cells (Malvern Instruments, Worcestershire, UK).

5.2.5 Lycopene concentration

The absorbance of lycopene containing emulsions was used to determine lycopene concentrations. Periodically, sample absorbances were measured using a Shimadzu UV-2101 PC UV-Vis Scanning Spectrophotometer equipped with an ISR – Integrating Sphere Assembly (Shimadzu, Kyoto, Japan). Lycopene content was determined using a standard curve created using mixtures of emulsions with and without added lycopene. Concentrations were expressed relative to zero time lycopene concentrations since small differences in initial lycopene concentrations occurred in the different emulsions. Duplicate samples were tested for each treatment at each testing time.

5.2.6 Hydroperoxide content of surfactants

Hydroperoxide concentrations of Tween 20 and Brij 35 were determined by a modified version of Nuchi et al. (103). Small amounts of pure surfactant were added to 2.8 ml of methanol/1-butanol (2:1, v/v). Thiocyanate/ferrous solution (30 μL) (prepared by mixing equal volumes of 0.144 M $FeSO_4$ •7 H_20 with 0.132 M $BaCl_2$ (acidic solution), centrifuging, and mixing equal volumes of the clear ferrous solution with 3.94 M ammonium thiocyanate) was added to the methanol/1-butanol mixture, vortexed, and incubated at room temperature for 20 minutes. Following the incubation period, sample absorbances were read at 510 nm using a Genesys 20 spectrophotometer

(ThermoSpectronic, Waltham, Massachusetts, USA). The hydroperoxide content was determined using a standard curve developed using known concentrations of cumene hydroperoxide.

5.2.7 Ferrous iron content of emulsions and surfactant solutions

Ferrous iron content was determined using ferrozine chelation, a method initially developed by Stookey (152). A ferrozine solution (3 mM final concentration) was added directly to emulsion samples and absorbance was measured at 562 nm using a Shimadzu UV-2101 PC UV-Vis Scanning Spectrophotometer equipped with an ISR – Integrating Sphere Assembly (Shimadzu, Kyoto, Japan). For experiments measuring the conversion of added ferric iron to the ferrous form, ferrozine was added to the emulsion at the time of ferric addition, and the development of the ferrozine -ferrous iron complex was monitored over time. For samples monitoring the conversion of ferrous iron to ferric species, ferrozine was added to the sample after different incubation times.

For examining the loss of ferrous iron in surfactant solutions, a concentrated stock solution of ferrous sulfate heptahydrate was added to surfactant solutions (0-30 mM surfactant) to give a final concentration of 100 uM ferrous sulfate. This mixture was vortexed and allowed to react for 10 minutes. After 10 minutes, ferrozine solution (3 mM final concentration) was added to the samples and absorbance was measured at 562 nm using a Genesys 20 spectrophotometer (ThermoSpectronic, Waltham, Massachusetts, USA). Emulsion and surfactant solution ferrous iron concentration was determined using standard curves prepared from ferrous sulfate.

5.2.8 Statistical analysis

All measurements were made using triplicate samples. Statistical Analysis Systems Version 9.1 software (SAS Institute, Cary, NC, USA, 2002) analysis of variance procedures (PROC GLM combined with the LS MEANS, SLICE and PDIFF functions) were used to analyze results. In this analysis, surfactant type, iron addition, chelator, and storage time were considered fixed effects. Where significant interactions were found among the effects tested, a Bonferroni adjustment ($p \leq 0.05$/# of comparisons) was used for declaring significance.

5.3 Results and discussion

5.3.1 Stability of anionic and nonionic surfactants

Three small molecule surfactants, SDS, Brij 35, and Tween 20, were used to stabilize emulsions with an oil phase comprised of lycopene dispersed in hexadecane. Hexadecane was chosen due to its high stability towards lipid oxidation (148). Previous work (159) has shown that SDS-stabilized lycopene in hexadecane does not exhibit the development of lipid oxidation products, suggesting that hexadecane is not degrading and that lycopene degradation in this system is likely due to mechanisms other than the reaction with radical species produced during oxidation of the bulk component of the oil phase. Work with SDS-stabilized lycopene in hexadecane emulsions has also shown that transition metals, and to a lesser extent, free radicals, play a substantial role in the degradation of lycopene in these systems (158). The goal of this research was to further understanding of lycopene degradation in oil-in-water emulsions by using nonionic surfactants in place of anionic SDS.

In this work, lycopene stability was found to be significantly higher in oil-in-water emulsions stabilized with Tween 20 or Brij 35 than emulsions stabilized by SDS (**Figure 5.1**). Previous studies (104, 111, 112) have found that emulsions stabilized by anionic surfactants oxidized at the fastest rates, followed by nonionic surfactants, while cationic surfactant-stabilized emulsions exhibited the slowest oxidation. These results were attributed to the fact that cationic surfactants create a positively charged barrier around the oil droplets that repel cationic iron and other metals, while anionic surfactants attract iron to the droplet surface where it can easily degrade oil phase components. The larger negative charge present on the surface of SDS-stabilized droplets (average zeta potential = 174) is likely to more strongly attract transition metals (present in trace amounts in surfactants, buffer components, etc.) to the surface of the droplets than Brij 35 or Tween 20 (average zeta potential = -3.2 and -4.3). Once in contact with the droplet surface, transition metals like ferric iron may be capable of reacting with lycopene to produce lycopene radical cations (69, 70, 72-74). These radical cations may participate in additional lycopene degradation pathways, eventually leading to substantial losses of lycopene as seen in **Figure 5.1**.

Relatively small differences were seen in the loss of lycopene between the two nonionic surfactants. No significant differences were found between the Tween 20 and Brij 35 treatments until 50 hours of storage, at which time the Brij 35 and Tween 20 emulsions had lost 22 and 29% of their initial lycopene respectively. At later testing times, lycopene stability was slightly lower in the Tween 20 than the Brij 35 stabilized emulsions.

5.3.2 Ferric iron addition

The addition of 100 uM ferric chloride (**Figure 5.2**) resulted in a decrease in stability for both Tween 20 and Brij 35-stabilized emulsions. Compared to emulsions with no added iron (**Figure 5.1**), Tween 20 treatments experienced a 25% greater loss of lycopene after 95 hours when 100 uM ferric chloride was present. The Brij 35 emulsions experienced a slightly greater lycopene degradation in the presence of ferric chloride, with 35% less lycopene present than the no iron control at 95 hours of storage. Addition of ferric chloride resulted in fairly similar degradation patterns between these two nonionic surfactants. For the first two hours of storage, the Tween 20 experienced significantly greater loss of lycopene than the Brij 35 samples. However, after this testing time, no significant differences were found between the two treatments until 95 hours of storage.

The results of ferric chloride addition in Brij 35 and Tween 20 emulsion systems were found to be quite different from results found previously in lycopene emulsions stabilized by SDS (158). In SDS emulsions, ferric addition resulted in a much more rapid loss of lycopene, with over 80% of the lycopene destroyed within the first hour of storage. This rapid degradation was proposed to have been due to the reaction of ferric ions with neutral lycopene molecules to produce lycopene radical cations and ferrous ions (Equation 1). In this previous work, further evidence of this reaction was provided by using ferrozine to monitor the development of ferrous ions in SDS emulsions with or without lycopene present. When lycopene was present over 90% of the ferric iron was converted to the ferrous form within 300 seconds, while iron reduction did not occur when lycopene was not present. Ferric chloride addition was also found to produce

greater degradation than controls in lycopene dispersed in bulk hexadecane (158). The differences in rate of lycopene loss between the anionic SDS and nonionic Brij 35 and Tween 20-stabilized emulsions may again be due to the greater attractive forces between the highly negative charges on the emulsion droplets stabilized by SDS bringing the ferric ions into closer proximity to the oil droplets where contact with lycopene can occur.

5.3.3 Ferrous iron addition

The addition of 100 uM ferrous sulfate resulted in the most rapid decrease in stability of all the treatments for both Tween 20 and Brij 35. Compared to emulsions with no added iron (**Figure 5.1**), Brij 35 and Tween 20 treatments experienced a 61 and 34% greater loss of lycopene respectively after 95 hours when 100 uM ferrous sulfate was present (**Figure 5.3**). The Brij 35 treatments exhibited the most dramatic decrease, with 68% of the initial lycopene present being lost within the first hour of storage compared to a 13% loss within the first hour of Tween 20 emulsion storage.

The rapid degradation of lycopene found in the Brij 35 and Tween 20 treatments was not found when ferrous sulfate was added at the same concentration to SDS-stabilized emulsions or hexadecane with added lycopene (158). One reason for this difference might be the presence of hydroperoxides in the nonionic surfactants. Both Brij 35 and Tween 20 are known for developing hydroperoxides over time (102). Hydroperoxides can react with iron to form highly reactive peroxyl and alkoxyl radicals (Equations 5.2 and 5.3)(102, 105). Ferrous iron (Fe^{2+}) is much more reactive than ferric iron (Fe^{3+}), resulting in Reaction 5.3 proceeding at a rate 10^7 times faster than Reaction 5.2 (106).

$$Fe^{3+} + ROOH \rightarrow Fe^{2+} + ROO\bullet + H^{+} \quad (5.2)$$

$$Fe^{2+} + ROOH \rightarrow Fe^{3+} + RO\bullet + OH^{-} \quad (5.3)$$

If hydroperoxides are present in the surfactant system, it might be possible for these reactions to take place at the oil droplet surface. If such a reaction is occurring in the emulsions treated with ferrous sulfate, it might be possible to for the ferrous form to quickly be converted to the ferric form at the emulsion droplet interface. Once this conversion has taken place, the newly formed ferric species as well as the alkoxyl radical might be in close proximity to the lycopene present in the oil droplet and might be able to react with lycopene to produce both transition metal and radical-initiated degradation pathways. To examine this hypothesis further, several experiments were conducted to explore hydroperoxide levels in the system and the ability of ferrous and ferric iron to convert to other states within the emulsion system.

5.3.4 Hydroperoxide levels

Since hydroperoxides are known to develop in Brij and Tween surfactants (102), the pure surfactants were tested to examine whether the presence of these reactive groups might provide some explanation of the ferrous iron results. The results of the hydroperoxides levels in the pure surfactant were then calculated to what level this would equate to in the 30 mM surfactant solution used as the aqueous phase of the emulsion. As seen in **Figure 5.4**, the average hydroperoxide level in the Brij 35 samples was 3.8 uM, which was much lower than the 202.4 uM level found in Tween 20 samples. These results give some plausibility to the theory that the ferrous iron could be converted to ferric iron by reacting with hydroperoxides. However, one aspect that cannot be

explained by these results is why the lycopene stability is greater in the Tween 20 treatments even though Tween 20 has much higher hydroperoxide levels than Brij 35.

5.3.5 Conversion of ferrous sulfate by surfactant solutions

Additional information on the influence of surfactants on the state of added iron was gained by combining 100 uM ferrous sulfate with various concentrations of surfactant solutions (0-30 mM surfactant). As seen in **Figure 5.5A**, when no or low concentrations of surfactant are present, nearly all of the iron remains in the ferrous state. However, as surfactant concentration was increased, much of the iron was converted to a state other than ferrous, which is the form complexed in the ferrozine reaction. At the 30 mM concentration, the concentration of surfactant used in preparing emulsions, nearly all of the iron in both Brij 35 and Tween 20 treatments has been converted to a form other than ferrous. At the 30 mM surfactant concentration, there is also no significant difference between the ferrous iron concentrations in the Brij 35 and Tween 20 treatments. Again, this conversion is likely due to interaction with peroxides in the surfactant. **Figure 5.5B** shows that when various concentrations of hydrogen peroxide are combined with 100 uM ferrous sulfate, more ferrous iron is converted as hydrogen peroxide concentration is increased. When hydrogen peroxide is present at a near equal concentration, nearly all of the ferrous ions have been converted.

Questions remain as to the mechanism by which the 30 mM Brij 35 solution causes the loss of ferrous iron given the low levels of hydroperoxides present in the pure surfactant samples. It might be possible that additional peroxides developed during the time in which the ferrous sulfate and Brij 35 solutions were allowed to react. Since the

reaction described in Equation 3 produces a radical species, it might be possible that this radical could react with additional groups on Brij molecules to produce additional peroxides, which could react with additional ferrous ions. This might occur with relative ease given the linear nature of Brij 35 (linear formula = $C_{12}H_{25}(OCH_2CH_2)_{23}OH$), which would likely have little stearic hindrance. Previous work on interactions between iron and surfactant micelles has also shown a concentration dependent development of peroxides in surfactants upon exposure to ferrous ions (102).

5.3.6 Ferrous iron concentrations in emulsions with and without lycopene

Work with ferrozine was also used to attempt to understand the interaction of ferrous and ferric iron within the emulsion system. Emulsions were prepared with both Brij 35 and Tween 20 as surfactants. For each surfactant, emulsions were made with and without lycopene in the oil phase. Once emulsions were prepared, concentrated stock solutions of either ferric chloride or ferrous sulfate were added to the emulsion samples. ferrozine was added to the emulsions to determine the concentration of ferrous iron present in the emulsions at a given storage time.

When ferrous sulfate was added to emulsions stabilized by Tween 20, nearly all of the ferrous iron present (~98%) was converted within 5 minutes of storage (**Figure 5.6A**). The rate of ferrous iron conversion was similar between the emulsions made with and without lycopene, with significant differences found between these two treatments only at the 0 and 30 second testing times. When ferrous sulfate was added to Brij 35 emulsions, the conversion of ferrous iron was slower than that found in the Tween 20 samples (**Figure 5.7A**). However, nearly all of the ferrous iron had been converted

within the first hour of storage for the emulsion without lycopene, and within two hours of storage for the emulsion with lycopene. With the exception of the ferrous iron concentration present after five minutes of storage, the Brij 35 emulsions with and without lycopene were significantly different from one another until two hours of storage, when nearly all ferrous iron was lost in both systems.

When ferric chloride was added to Tween 20 emulsions with and without lycopene, 9-11% of ferric chloride was converted to the ferrous species over the seven hour storage time (**Figure 5.6B**). Similar to the results found in the samples with added ferrous sulfate, there were no significant differences in the development of ferrous species between Tween 20 emulsions with and without added lycopene. Significant differences were found over the entire storage time between emulsions with and without added lycopene when emulsions were stabilized by Brij 35 and 100 uM ferric chloride was added (**Figure 5.7B**).

The results of this set of experiments suggest that redox cycling may be occurring in Brij 35 emulsions with added lycopene, but not in Tween 20 emulsions. Since lycopene can react with the ferric form of iron to regenerate the ferrous species, it is not surprising that the presence of lycopene might result in the regeneration of ferrous iron. The slower loss of ferrous iron seen when lycopene is present in Brij 35 emulsions (**Figure 5.7A**) may be a result of ferric iron regeneration to ferrous by this reaction (Equation 5.1).

The lack of difference in ferrous iron content over time in the Tween 20 emulsions with and without lycopene suggests that lycopene-promoted redox cycling may not be occurring in the Tween 20 system. It is unclear why the cycling is not found

in this system. One reason may be that when the ferrous iron is converted to the ferric species, it is not in a location in which it can easily attack lycopene in the droplet. Tween 20 has a more branched structure than Brij 35 that may hinder the movement of the iron and may therefore slow the rate at which iron can reach the oil phase lycopene. There may also be differences in the partitioning of iron or lycopene into surfactant micelle structures within the emulsion since both Brij 35 and Tween 20 are used at concentrations higher than the critical micelle concentration (160). Previous work has shown that the partitioning of iron and hydroperoxides into aqueous phase micelles is impacted by the concentration of surfactant used and can impact the rate of lipid oxidation reactions (161). The ease of iron redox cycling taking place is therefore likely to be impacted by the presence of micelle structures, which are likely to have different properties depending on the surfactant type.

The redox cycling found in the Brij 35 emulsion containing lycopene may help to explain why there are such large differences in the rate of lycopene loss between Tween 20 and Brij 35 emulsions when ferrous sulfate is added to the system (**Figure 5.3**). If redox cycling is occurring in the Brij 35 emulsions, it is likely that lycopene is being attacked by both the ferric form of iron and the alkoxyl radical generated from ferrous iron-hydroperoxide interactions. With degradation occurring by two different mechanisms, it would be expected that lycopene loss would be more rapid in a system exhibiting redox cycling.

While the data on the conversion of iron species helps to explain some of the differences in lycopene stability in the nonionic emulsion systems, a question that still remains is why the ferric chloride treated emulsions do not exhibit as much lycopene

degradation as those treated with ferrous sulfate assuming that most of the ferrous ions in the ferrous sulfate treatments are converted to the ferric form by hydroperoxides. Ferric chloride does have an impact on stability, but it is clearly less active than ferrous sulfate as evidenced both by the higher stability of lycopene in ferric chloride treated emulsions and the relatively low conversion of ferric ions to ferric species in the ferrozine chelation experiments reported in **Figures 5.6B and 5.7B**. One reason for the differences between the ferric species generated from ferric chloride and those generated from ferrous sulfate reacting with hydroperoxides may be the location of these species within the emulsions. In ferrous sulfate treatments, the ferric species are likely generated at the emulsion droplet surface because this is the location for much of the hydroperoxide containing surfactants. When generated at the droplet interface, the ferric species are already in close proximity to lycopene, facilitating degradative reactions. On the other hand, ferric species introduced into the emulsion system in the form of ferric chloride solutions, may not be in such close proximity to lycopene. There may be differences in the partitioning of ferrous sulfate and ferric chloride within the structures of the emulsion system. Solubility may also be a factor since the ferrous species of iron has a solubility 10^{13} times greater than ferric species (155). There may also be differences in the relative ease at which ferrous and ferric species may come into contact with the droplet interface since the effective radius of ferric species in aqueous solutions is 0.9 nm, while ferrous species have a smaller effective radius of 0.6 nm. This slightly smaller radius may facilitate contact with the droplet surface for ferrous ions, which can then oxidize to the ferric state and damage lycopene.

5.4 Conclusion

This research has given further evidence that surfactant type can greatly impact the stability of oil phase components in oil-in-water emulsions. Experimental results comparing anionic and nonionic surfactants provide additional support to previous studies that have suggested that anionic surfactants create more rapid degradation reactions than nonionic surfactants due to their ability to attract transition metals to the droplet interface. This work has also shown that unlike SDS-stabilized emulsions, the addition of ferrous sulfate to Tween 20 and Brij 35 emulsions can create rapid degradation of lycopene. Some questions still remain on the exact mechanisms by which some of the degradation patterns occurred, however, it is likely that ferrous iron activity can be attributed to the presence of hydroperoxides in the nonionic surfactants that can oxidize the ferrous iron to the ferric species that can cause lycopene degradation. The results of this study also suggest that the breakdown of hydroperoxides may also lead to radical-initiated lycopene degradation pathways. More work is needed to fully understand the differences seen between the Tween 20 and Brij 35 emulsions. However, the evidence produced in this work suggests that hydroperoxide content of surfactants should be carefully considered in the formulation of emulsion delivery systems for carotenoids.

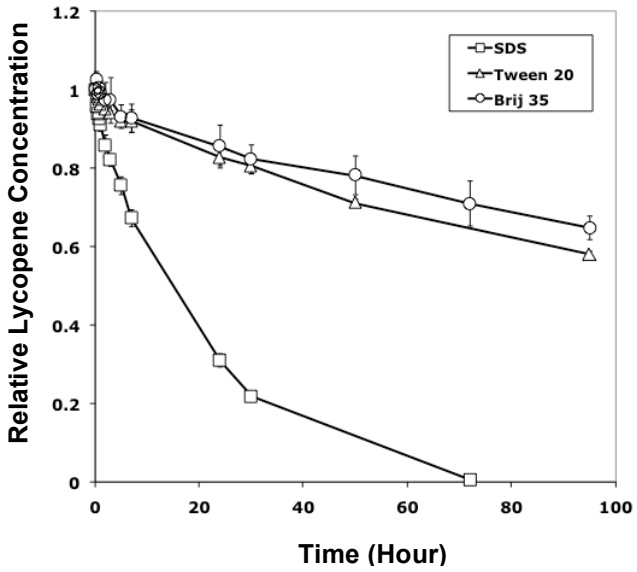


Figure 5.1: Relative lycopene concentration over storage time in SDS, Tween 20, and Brij 35-stabilized emulsions at pH 3.0 with no added iron. Data points represent means (n=2) $\pm$ standard deviations. Some error bars lie within the data points.

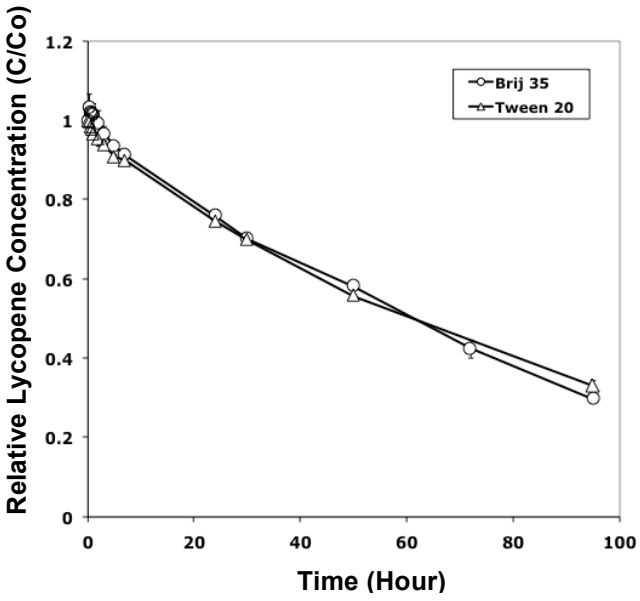


Figure 5.2: Relative lycopene concentration over storage time in Tween 20 and Brij 35-stabilized emulsions with 100 uM ferric chloride. Data points represent means (n=2) $\pm$ standard deviations. Some error bars lie within the data points.

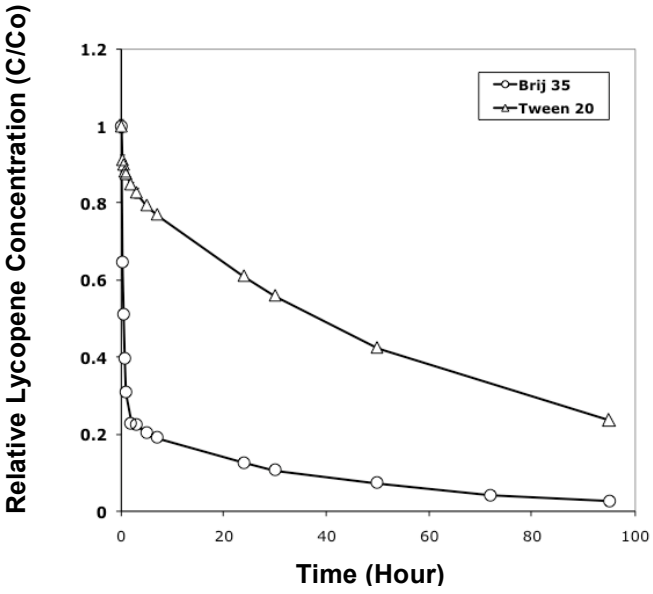


Figure 5.3: Relative lycopene concentration over storage time in Tween 20 and Brij 35-stabilized emulsions with 100 uM ferrous sulfate. Data points represent means (n=2) $\pm$ standard deviations. Some error bars lie within the data points.

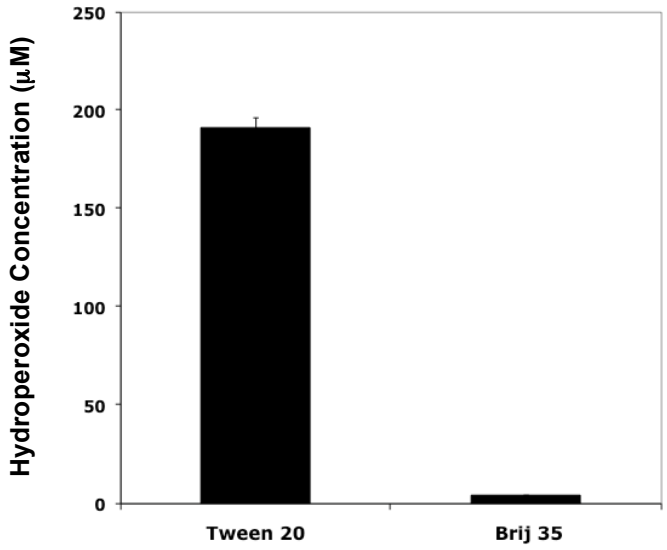
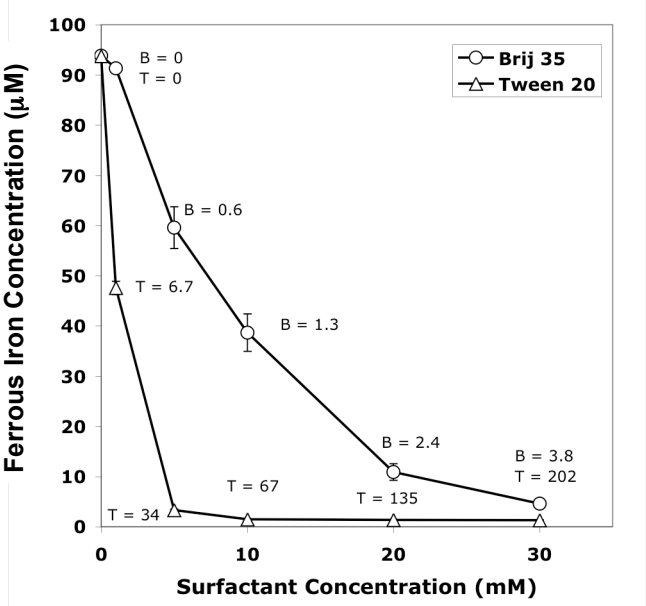


Figure 5.4: Hydroperoxide concentration of 30 mM Tween 20 and Brij 35 solutions. Data points represent means (n=6) ± standard deviations.

(A)



(B)

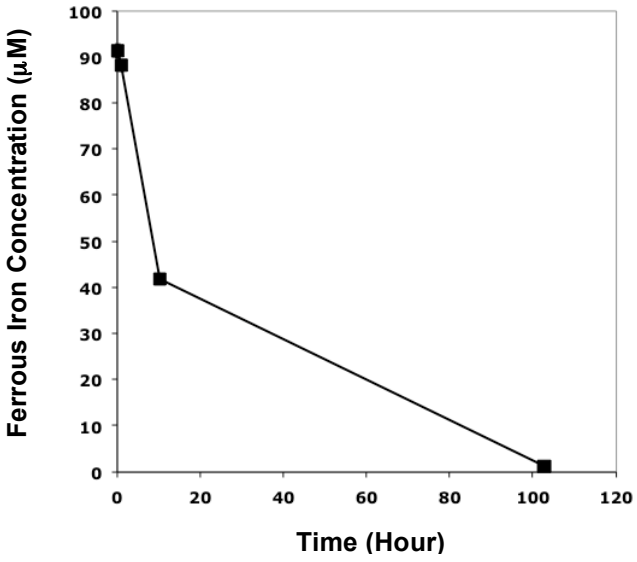
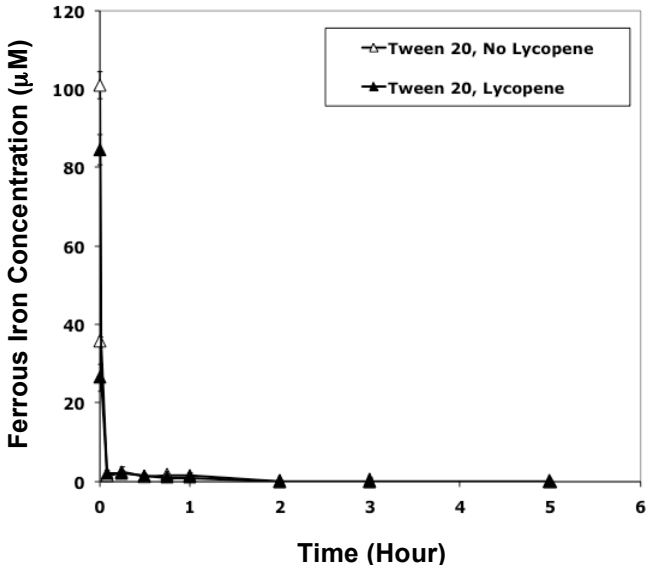


Figure 5.5: Ferrous iron concentration of (A) Tween 20 and Brij 35 surfactant solutions, or (B) hydrogen peroxide solutions, 10 minutes after the addition of 100 uM ferrous sulfate. Labels beside data points indicate the concentration of hydroperoxides calculated to be present in the surfactant solution. Data points represent means (n=3) $\pm$ standard deviations. Some error bars lie within the data points.

(A)



(B)

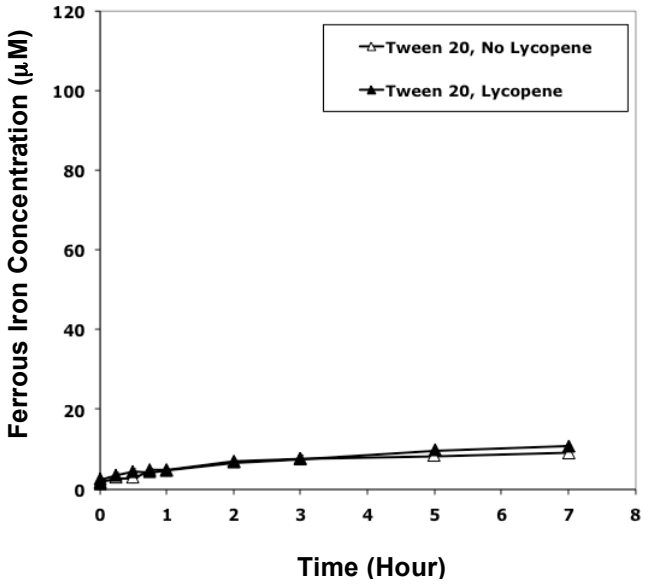
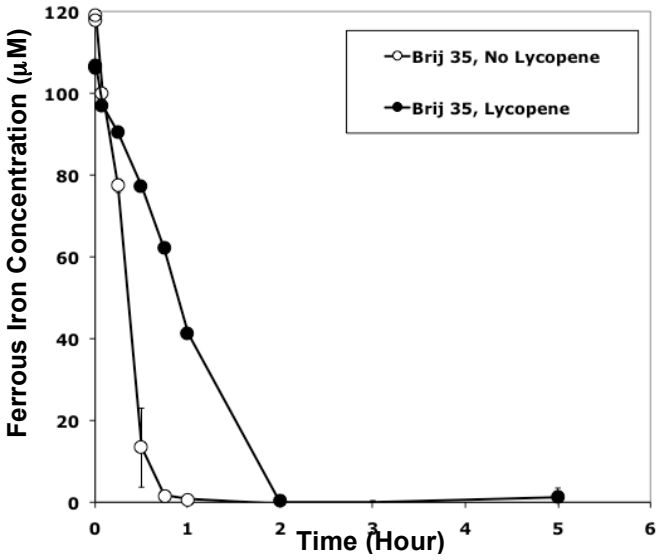


Figure 5.6: Ferrous iron concentration over time in Tween 20-stabilized emulsions with and without lycopene after the addition of (A) 100 uM ferrous sulfate, or (B) 100 uM ferric chloride. Data points represent means (n=2) $\pm$ standard deviations. Some error bars lie within the data points.

(A)



(B)

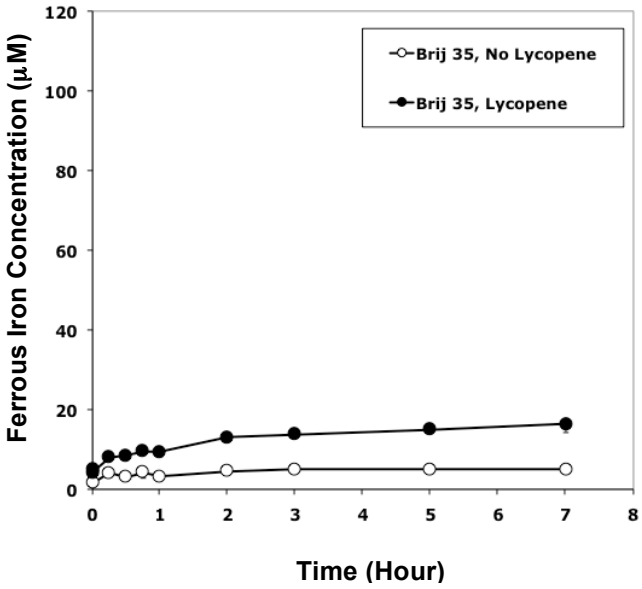


Figure 5.7: Ferrous iron concentration over time in Brij 35-stabilized emulsions with and without lycopene after the addition of (A) 100 uM ferrous sulfate, or (B) 100 uM ferric chloride. Data points represent means (n=2) $\pm$ standard deviations. Some error bars lie within the data points.

CHAPTER 6

FUTURE WORK

While this body of research has made progress towards developing a stable emulsion delivery system for lycopene and understanding major mechanisms of lycopene degradation in these systems, there are several areas in which additional work is needed. Potential areas of future work include conducting additional work to clarify current results, employing additional strategies to improve the oxidative stability of the system, understanding the efficacy of lycopene enriched functional foods, and applying the knowledge of lycopene degradation mechanisms to nutrition research studies on carotenoids.

Several questions remain on the results found in this study. One of these questions surrounds the findings that corn oil emulsion stabilized with Brij 35 showed similar or greater stability than DTAB-stabilized emulsions. In previous studies (104, 105, 111, 114, 131, 134) emulsions stabilized by cationic surfactants, like DTAB, have shown greater stability than nonionic surfactants, like Brij 35. Interfacial thickness and the relative ability of certain surfactant micelles to partition proxidants may be reasons for these differences, but more work is needed to fully understand this phenomenon.

Other questions remain on the results found in the final portion of this work. More work is needed to understand why ferrous iron addition to emulsions stabilized with nonionic surfactants results in rapid degradation of lycopene while having little effect or an antioxidative effect in SDS-stabilized emulsions and in bulk oil. Further

work will likely need to explore the potential for peroxides to convert ferrous iron to the ferric form and potential differences in reactivities and locations of peroxides and iron.

A second area for future work would be to attempt additional strategies for physically and chemically stabilizing lycopene emulsions. One strategy might be to use proteins as surfactants. Proteins may offer the beneficial characteristics of both being label friendly emulsifiers and acting as chelators and free radical scavenging antioxidants. An additional strategy for improving lycopene stability in emulsion systems may be to add additional antioxidants to the system that could serve to protect lycopene. Free radical scavenging antioxidants and chelators could be added to the system to provide multiple means of preventing degradation.

In addition to improving the emulsion system for use in food products, much work is needed to understand the bioavailability and function of the lycopene contained in this system once it enters the body. Some theories suggest that carotenoids may be more bioavailable when lipids are present and when the plant cellular matrix is broken down by processing to release more chromoplast carotenoids (5, 14, 39-44, 44-46). Since the lycopene in this system is not trapped by a cellular matrix and since it is dispersed in oil, it is possible that it is highly bioavailable. However, human or cell culture studies would need to be performed to confirm this. A related area of future work would be to study the isomeric composition of lycopene in the emulsion system and how this might be changed with thermal processing. Some evidence suggests that *cis* forms of lycopene might be more bioavailable or bioactive than the *trans* form (14, 32, 33, 35, 36). High performance liquid chromatography (HPLC) could be used to study differences in isomeric composition in these systems. It would also be useful to attempt to incorporate

other carotenoids (lutein, zeaxanthin, beta-carotene) into the emulsion delivery system and study differences in stability, bioavailability, and bioactivity of these carotenoids compared to lycopene.

Finally, it may be critical to consider the findings of this work in designing nutritional studies on carotenoid activity of foods and nutritional supplements. This research has clearly shown that the presence of transition metals and to a lesser extent, free radicals, can greatly decrease the stability of lycopene in the system, leading to degradation products and probably loss of beneficial bioactivity. Some degradation products might even have detrimental effects in the body, as they may be radical species. Given the rapid degradation seen in this research, it may be important for those designing nutritional studies to consider the effects of storage and the presence of trace metals in supplement processing on the carotenoid being tested. Otherwise, there is potential that the carotenoid being tested may be significantly degraded over the course of the study.

CHAPTER 7

CONCLUSION

This body of work examined the potential for using oil-in-water emulsions as delivery systems for lycopene into functional foods. Emulsions were successfully prepared using lycopene dispersed both in corn oil and hexadecane as the oil phase. Two methods of preparation, by sonication or using a two-stage homogenizer, were found to produce stable emulsions using a variety of surfactants (SDS, Brij 35, DTAB, and Tween 20).

In all emulsions without added antioxidants, lycopene degradation was fairly rapid, with significant losses of lycopene within a few days, or in some cases, a few hours, even at low storage temperatures (15 or 25ºC). Given this instability, work was conducted in an effort to understand the mechanisms by which lycopene degradation in this system. Initial work suggested that both free radicals and contact with transition metals might be involved. The presence of minor components of corn oil (i.e. tocopherol) was found to improve the stability of lycopene compared to corn oil stripped of these components and hexadecane. These minor components are known for their abilities to scavenge free radicals, and their protective attributes in this system suggest a free radical degradation pathway. The fact that lycopene degradation occurred even in the absence of the development of reactive lipid oxidation products in emulsions prepared with hexadecane, also suggests that an additional pathway of lycopene degradation may be occurring.

Further understanding of the mechanisms of lycopene degradation in emulsion systems was gained by using a model emulsion system using hexadecane, a highly stable

saturated hydrocarbon, as the major component of oil phase. In this work, common initiators of carotenoid degradation were tested to evaluate their relative impact on lycopene stability in emulsions. Light was found to have little impact on lycopene stability. Lowering the pH of the emulsion decreased the stability of lycopene. Addition of metal chelators, and to a lesser extent addition of free radical scavengers improved the stability of lycopene, suggesting that metals and free radicals are involved in degradation pathways.

The role of transition metals in lycopene degradation was explored further by adding ferrous and ferric iron to the emulsions. In SDS-stabilized emulsions and in bulk hexadecane with added lycopene, ferric iron was found to create rapid degradation, while ferrous iron was found to have little effect or to actually increase the stability of lycopene. In emulsions stabilized by the nonionic surfactants Brij 35 and Tween 20, ferric addition also led to lycopene degradation, but to a lesser degree than in SDS-stabilized emulsions. Ferrous iron was found to have a greater destabilizing effect in the emulsion stabilized by nonionic surfactants. The reason for this activity of the ferrous iron in the nonionic emulsions, but not in the ionic, SDS-stabilized emulsions is not completely clear. However, there is some evidence to suggest that naturally present hydroperoxides present in the nonionic surfactants may play a role in converting ferrous iron to the ferric species, which could then attack lycopene. This reaction also results in the production of radical species produced from the breakdown of the hydroperoxide, which can also attack lycopene.

Overall, the results of this work suggest that emulsions have potential as delivery systems for carotenoids like lycopene into foods. These emulsions would be especially

useful delivery systems if additional components like metal chelators and free radical scavengers are added to provide protection to the carotenoid and if the surfactant used to produced the emulsion was selected based on its ability to repel transition metals and provide a thick interfacial barrier to aqueous phase proxidants. This work also suggests that food formulation and process development should take into account the role of iron in carotenoid degradation.

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